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Characterization of the sea bass melanocortin 5 receptor: a putative role in hepatic lipid metabolism

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SUMMARY

The melanocortin 5 receptor (MC5R) plays a key role in the regulation of exocrine secretion in mammalian species. This receptor has also been characterized in some fish species but its function is unknown. We report the molecular and pharmacological characterization, as well as the tissue expression pattern, of sea bass MC5R. Cloning of five active alleles showing different levels of sensitivity to endogenous melanocortin and one non-functional allele demonstrate the allelic complexity of the MC5R locus. The sea bass receptor was activated by all the melanocortins tested, with ACTH and desacetyl-MSH and β-MSH showing the lowest efficiency. The acetylation of the MSH isoforms seems to be critical for the effectiveness of the agonist. Agouti-related protein had no effect on basal or agonist-stimulated activation of the receptor. SbMC5R was mainly expressed in the brain but lower expression levels were found in several peripheral tissues, including liver. Progressive fasting did not induce up- or downregulation of hypothalamic MC5R expression, suggesting that central MC5R is not involved in the regulation of food intake in the sea bass. MTII, a sbMC5R agonist, stimulated hepatic lipolysis *in vitro*, measured as free fatty acid release into the culture medium after melanocortin agonist exposure of liver fragments, suggesting that MC5R is involved in the regulation of hepatic lipid metabolism. Taken together, the data suggest that different allelic combinations may confer differential sensitivity to endogenous melanocortin in tissues where MC5R is expressed and, by extension, in hepatic lipid metabolism.

Key words: melanocyte-stimulating hormone (MSH), proopiomelanocortin (POMC), MC5R, phosphodiesterase inhibitor (IBMX).

INTRODUCTION

Melanocortins are produced by posttranscriptional processing of the proopiomelanocortin (POMC) precursor. In tetrapods, melanocortins comprise three different melanocyte-stimulating hormones (α-, β- and γ-MSH) and adrenocorticotropic hormone [ACTH (Castro and Morrison, 1997]. Teleost fish lack γ-MSH and the POMC gene encodes an extra MSH (δ-MSH) in elasmobranches (Takahashi and Kawauchi et al., 2006). Melanocortin signalling is mediated by binding to a family of specific G-protein-coupled receptors (GPCR) that positively couple to adenylyl cyclase. Similarly to the agonist system, the number of receptors diverges in vertebrate species. Tetrapod species invariably have five melanocortin receptors (MC1R–MC5R). However, zebrafish has six MCRs, with two copies of MC5R, whereas pufferfish have only four receptors with no MC3R and only one copy of MC5R (Logan et al., 2003). MC2R is specific for ACTH whereas the four other MCRs distinctively bind MSHs (reviewed by Schiöth et al., 2005). Atypically, melanocortin signalling is not exclusively regulated by binding of endogenous agonists, as naturally occurring antagonists, agouti and agouti-related protein (AGRP), compete with melanocortin peptides by binding to MCRs. Agouti protein is a potent melanocortin antagonist at MC1R and MC4R. By contrast, AGRP is potent in inhibiting melanocortin signalling at MC3R and MC4R, but is not active at MC1R (Cone, 2006). Studies in mammalian species have demonstrated that MC1R is mainly involved in the control of skin pigmentation whereas MC2R regulates adrenal corticosteroid synthesis and secretion. MC3R and MC4R are mainly expressed in the central nervous system and regulate energy balance. MC5R has a wide distribution in the brain and peripheral tissues, but always shows low expression levels (Schiöth et al., 2005; Cone, 2006). Deletion of the MC5R gene in mice results in nearly total loss of ^{125}I-NDP-MSH binding sites in skeletal muscle and the Haderian, lachrymal and preputial glands, indicating that MC5R represents the major MCR in these tissues. MC5R knockout mice exhibit a severe dysfunction of exocrine secretion, affecting hair follicle-associated sebaceous, Harderian, lachrymal and preputial glands. The absence of MC5R expression results in reduced hair lipid content, which provokes defects in water repulsion, and reduced coat insulation against cold environments, resulting in an impaired thermoregulatory function (Chen et al., 1997).

Several studies have corroborated similar functions of fish melanocortin receptors to those reported in mammalian systems. Recent experiments have demonstrated the involvement of MC1R in the colour patterns of fish (Gross et al., 2009), the participation of MC2R in the regulation of cortisol secretion (Metz et al., 2005; Aluru and Vijayan, 2008) and the contribution of MC4R to the control of food intake (Cerdá-Reverter et al., 2003a; Sánchez et al., 2009a). However, MC3R and MC5R functions remain elusive in fish. Unlike in the mammalian system, MC5R is highly expressed within the telencephalon and hypothalamus of goldfish (Cerdá-Reverter et al., 2003b) and fugu (Klovins et al., 2004), suggesting its involvement in the regulation of an array of physiological functions. Despite the relative lack of knowledge of the function of fish MC5R, a few reports have focused on the characterization of this receptor (Ringholm et al., 2002; Cerdá-Reverter et al., 2003b; Klovins et al., 2004). In this paper, we report the molecular and pharmacological characterization of the

sea bass MC5R (sbMC5R). The results provide the first evidence of the complex allelic composition of the *MC5R* locus, as well as the existence of null alleles producing nonfunctional proteins and differences in the MC5R isoforms as derived form their sensitivity to endogenous melanocortin agonists. In addition, our data provide the first evidence of the involvement of fish MC5R in the control of hepatic lipid metabolism.

MATERIALS AND METHODS

Animals and reagents

Male and female sea bass (*Dicentrarchus labrax* L.) were kindly supplied by Tinamenor (Santander, Spain). Prior to the experiments, the animals were maintained in 500 l tanks supplied with continuously aerated running sea water for 2 months. Fish were hand fed at 09:00 h with a commercial diet (Proaqua Nutrición, Palencia, Spain). Animals were anesthetized in 2-phenoxy-ethanol (0.1%) for 2 min before any manipulation and killed by rapid decapitation. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/CEE) concerning the protection of experimental animals. Melanocortin peptides were from Bachem (Germany). Zebrafish agouti-related protein (zfAGRP), was kindly donated by Dr Millhauser from Department of Chemistry, University of California, Santa Cruz, USA (Sánchez et al., 2009a). Unless otherwise indicated, all reagents were purchased from Sigma (St Louis, MO, USA).

Molecular cloning of sea bass MC5R

Genomic DNA isolated from blood was used as template for touchdown PCR reactions with Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and degenerate primers designed against conserved regions of the known MCR sequences. The 5′ primer (MCR3Fw2) was a 20-mer with the sequence: 5′-TACCACAGYATCRTGACMGT-3′. The 3′ primer (RevFish) had the sequence 5′-TSAGVGTGATGGCKCCCTT-3′ (Fig. 1). PCR products of about 274 base pair (bp) were isolated from low melting point (LMP) Nusieve GTG agarose gel (FMC) ligated into pGEM-T easy vector (Promega, Madison, WI, USA) and subsequently transformed into XLI-Blue *E. coli*. One clone that contained an insert of the expected size was sequenced. The 3′ extreme was resolved by 3′ RACE PCR. Specific primers for RACE-PCR were sbMC5R_3RACE_1 (5′-GACAGTGAAGAGAGCCG-3′) and sbMC5R_RACE_2 (5′-TGCTATGCTGCTCATCATG-3′). A fragment of about 678 bp was subcloned into pGEM-T easy vector and sequenced. The 5′ region was cloned by nested PCR using a cDNA library as template (kindly supplied by Dr Bernardini form the University of Insubria, Italy), sbMC5R-specific (MC5R_5′ RACE_cDNA_synthesis: 5′-ACTCCTGAAGGCATAGAT-3′ and MC5R-5WALKER2: 5′-GAACATTGAGACCAGGCAGATGA-TGACG-3′) and universal PBSSK-vector primers. A fragment of about 812 bp was subcloned into pGEM-T easy vector and sequenced. Finally the coding region was amplified using specific primers (*Hin*d-sbMC5R-Forward 5′-TATAAGCTTATGAATG-CATCAGATGATCAGT-3′, *Xho*-sbMC5R-Reverse 5′-TTACT-CGAGTCATGTGGGCTTCTCCTCG-3′ and brain cDNA, synthesized from total RNA pooled from several animals, as template. Fragments of about 990 bp were subcloned into pGEM-T easy vector and sequenced on both strands. The nucleotide sequences of sbMC5Rs have been deposited in the EMBL Nucleotide Sequence Database under accession numbers: P0: FN429774, P1: FN429769, P2: FN429770, P5: FN429772, P8: FN429773.

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). Superscript II reverse transcriptase (Invitrogen) was used for cDNA synthesis by priming with oligo(dT)$_{12\text{-}18}$ (Invitrogen). The cDNA was subsequently used as template for touchdown PCR reactions with Taq DNA polymerase (Invitrogen) and specific primers. The 5′ primer was sbMC5R_3′ RACE1 (5′-GACAGTGAAGAGAGCCG-3′) and the 3′-primer was MC5R_5RACE_cDNA (see above). Subsequently, PCR fragments were separated on 1.2% agarose gel and transferred by capillarity to Hybond-N nylon membrane (Amersham, Piscataway, NJ, USA). 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^{-1} yeast tRNA type III; 1× SSPE contained 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 9 mmol l^{-1} NaH_2PO_4; pH 7.4). Hybridization was carried out overnight in fresh hybridization solution containing 0.5×10^6 c.p.m. ml^{-1} [α-^{32}P]dCTP at 42°C. A probe containing the central region of the sbMC5R 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. Touchdown PCR for 18S RNA was carried out as an internal control for the reverse transcription step. Primer sequences were 18S_Fw 5′-GCATGCCGGAGTCTCGTT-3′ and 5′ 18S_Rev 5′-TGCATGGCCGTTCTTAGTTG-3′.

Real-time quantitative PCR

To evaluate mRNA levels, total RNA from individual hypothalamus or pituitary was treated with RQ1-DNAse. One microgram was used as template for cDNA synthesis, which was primed with random hexaprimers (Invitrogen). Two microlitres of cDNA (*sbMC5R*) or diluted cDNA (18S RNA) and primers (70 nmol l^{-1}) were added to 7.5 µl of Sybr Green PCR master mix (ABgene, Thermo Scientific, Madrid, Spain) and the volume was adjusted to 15 µl with water. PCRs were carried out on an iCycler (Bio-Rad, Madrid, Spain). Data were analyzed with the ΔΔCt (cycle threshold) method. As internal control a fragment of the sea bass 18S RNA gene was amplified, using primers 18S_Fw and 18S_Rev primers (see above). Expression of 18S RNA gene was demonstrated to be stable after fasting in previous studies (Sánchez et al., 2009a). Specific gene primers were as follows: qPCR_MC5R_Fw1 (5′-ATGAACACC-ACAGAGGCTCA-3′) and qPCR_MC5R_Rev1 (5′-ATAGCA-TCCTGTGGACGAGT3′).

Cell culture and transfection

HEK cells were transfected using a modified calcium phosphate transfection method (Chen and Okayama, 1987) and grown in DMEM (Invitrogen) containing 10% foetal bovine serum (Invitrogen), penicillin (100 i.u. ml^{-1}) and streptomycin (100 µg ml^{-1}) in a humidified atmosphere of 5% CO_2 at 37°C.

Galactosidase enzyme assay

Galactosidase enzyme assays were performed as previously described (Sánchez et al., 2009a). Briefly, the medium was removed and 50 µl of lysis buffer containing 250 mmol l^{-1} Tris-HCl pH 8 and 0.1% Triton X-100 were added. After one round of freezing (–80°C) and thawing, 10 µl of the lysate were preserved for protein assays. Phosphate saline buffer (40 µl) containing 0.5% BSA and 60 µl of substrate buffer (1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KCl, 5 mmol l^{-1} β-mercaptoethanol and 200 mg ml^{-1} *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, which was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Pharmacological experiments

A HEK293 cell clone, stably expressing β-galactosidase under the control of vasoactive intestinal peptide promoter placed downstream of tandem repeats of cAMP responsive elements (CRE), was generated by co-transfection (50:1) of pCRE/β-galactosidase plasmid [kindly supplied by Dr R. Cone, Vanderbilt University Medical Center (Chen et al., 1995)] and the tgCMV/HyTK plasmid, which harbours a hygromycin resistance gene (Wellbrock et al., 1998). Cells were selected in medium containing 400 μg ml^{-1} 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^{-1} bovine serum albumin, BSA + 0.1 mmol l^{-1} isobutylmethylxanthine (IBMX), containing 10^{-6} mol l^{-1} forskolin for 6 h. The clone showing highest response to forskolin (Clon-Q) was selected for subsequent experiments (Sánchez et al., 2009a). The full coding region of the *sbMC5R* was released from pGEM-T easy vector (see above) and directionally subcloned into pcDNA3 (Invitrogen). Double stable clones expressing β-galactosidase and *sbMC5R* were made by transfecting Clon-Q with the latter construct using G-418 selection (800 μg ml^{-1}). Clones were tested by incubating cells with MTII 10^{-6} mol l^{-1} in the assay medium. The clone Q/P5-23 was selected for following characterization of the activation profiles in response to several melanocortins (monoacetyl-MSH or α-MSH, diacetyl-MSH, desacetyl-MSH or MSH, human ACTH, monkey β-MSH, zfAGRP, SHU9119 and HS024) in the absence of IBMX. The effect of zfAGRP on basal sbMC5R activity was studied in both the presence and absence of the phosphodiesterase (PDE) inhibitor.

To test the activity of the different *sbMC5R* polymorphisms, Clon-Q was transiently transfected with sbMC5R-P0, -P1, -P2, -P5 or -P8 and a construct carrying the luciferase gene under the control of a constitutive promoter (Muriach et al., 2008). After 24 h, cells were plated in 96-well plates (50,000 cells/well). Forty-eight hours post-transfection, cells were stimulated with several endogenous melanocortins, including α-MSH, diacetyl-MSH, desacetyl-MSH, human ACTH and monkey β-MSH ranging from 10^{-6} to 10^{-8} mol l^{-1}, as before. Measurements were normalized for the protein content, the luciferase activity and forskolin-induced galactosidase activity. Receptor activation assays were always performed in quadruplicate wells and repeated at least three times independently.

Effects of progressive fasting on melanocortin system

To evaluate the effects of fasting on hypothalamic gene expression, 10 groups of 10 fish each [body mass=117±1.54 g] were acclimatized for 1 week to individual 500 l aquaria and fed *ad libitum* at 9:00 h. After this acclimatization period, five groups were fed the same ratio, and the other five were fasted. One fish from each fed or fasted group was sampled at 12:00 h (3 h post-feeding in the case of the fed groups) at 1, 4, 8 and 15 and 29 days, respectively. Anesthetized fish were weighed and blood samples were obtained by puncture of the caudal vessels. Subsequently the fish were decapitated and the whole hypothalamus dissected for immediate total RNA extraction. RNA samples were kept at –80°C in 75% ethanol until cDNA synthesis for quantitative PCRs (see above).

Effects of melanocortin agonist on hepatic lipolysis

To evaluate the effects of melanocortin agonist on hepatic lypolysis, the animals were sacrificed and their livers carefully removed. Small liver slices (50–80 mg) were dissected and incubated in 1 ml HB medium (136.9 mmol l^{-1} NaCl, 5.4 mmol l^{-1} KCl, 0.81 nmol l^{-1} $MgSO_4$, 0.44 mmol l^{-1} KH_2PO_4, 0.33 mmol l^{-1} Na_2HPO_4, 5 mmol l^{-1} $NaHCO_3$, pH 7.6), containing 5 mmol l^{-1} glucose, 1.5 mmol l^{-1} $CaCl_2$ and 2.5% fat-free BSA for 60 min at 25°C. Subsequently, the medium was removed and slices were incubated with 1 ml of HBS containing 10^{-6} mol l^{-1} MTII. After 2 and 4 h at 25°C medium was removed for determination of non-esterified fatty acids (NEFA) using commercial kits (WAKO Diagnostics, Neuss, Germany), following the supplier's recommendations. Liver slices were mechanically homogenized for protein determination using the BCA protein assay kit (Pierce). Subsequently, dose–response studies were done by incubating liver slices with MTII in HB ranging from 10^{-6} to 10^{-9} mol l^{-1} for 4 h. Experiments were always performed in quadruplicate wells and repeated at least three times independently.

Data analysis and statistics

Sequence comparisons and alignments were performed using ClustalX. A phylogenetic tree was derived using public domain ClustalX, which uses the Neighbour-Joining method on a matrix of distances. The position of transmembrane domains was drawn according to secondary structure prediction for human MC1R (Haitina et al., 2007a). Receptor activation data were fitted using SigmaPlot software. Differences in the sensitivity of sbMC5R isoforms and *sbMC5R* expression levels were analyzed by one-way ANOVA (P<0.05).

RESULTS

Molecular cloning of sbMC5R

By means of RT-PCR and using degenerate primers designed against conserved regions of fish melanocortin receptor sequences, we cloned a 274 bp fragment showing high identity with the MC5R sequences reported in other vertebrate species. The 5′ and 3′ regions were resolved by regular PCR and 3′ RACE PCR using a cDNA library or brain cDNA as templates, respectively. The full-length cDNA sequence was amplified by PCR using specific primers and brain cDNA as template. Fig. 1 shows the full-length cDNA sequence of isoform 1 of the sbMC5R (P1), which was sequenced twice on both strands. The cloned fragment contains an open reading frame of 1080 bp that encodes a putative 360 amino acid protein with seven putative hydrophobic transmembrane domains (TMDs; Figs 1, 2). Similar to other melanocortin receptors, the sbMC5R orthologue has short extracellular (ECL) and intracellular (ICL) loops and has cysteine residues at positions 266, 279 and 285 (sea bass numbering), which are fully conserved in all melanocortin receptors (Fig. 2 and http://www.gpcr.org). The deduced amino acid sequence shares 64–82% identity with other MC5R sequences but less than 59% with other MCR sequences. The identity is unequally distributed, and the N-terminal extracellular domain has the lowest similarity to other MC5Rs, including pufferfish MC5R. More than 50% of the divergence between sea bass and pufferfish MC5R orthologues resides within the N-terminal domain, encoding only 14% of the total protein length. A more detailed comparison among cloned fish MC5R shows that the overall identity level of sea bass receptor with other MC5Rs is highest (>96%) within TMD7 and lowest in TMD4 (<68%). The overall identity ranged between 80% and 92% within TMD1, TMD2, TMD3, TMD5 and TMD6. Unlike other MC5Rs, the sea bass protein has an extended intracellular tail

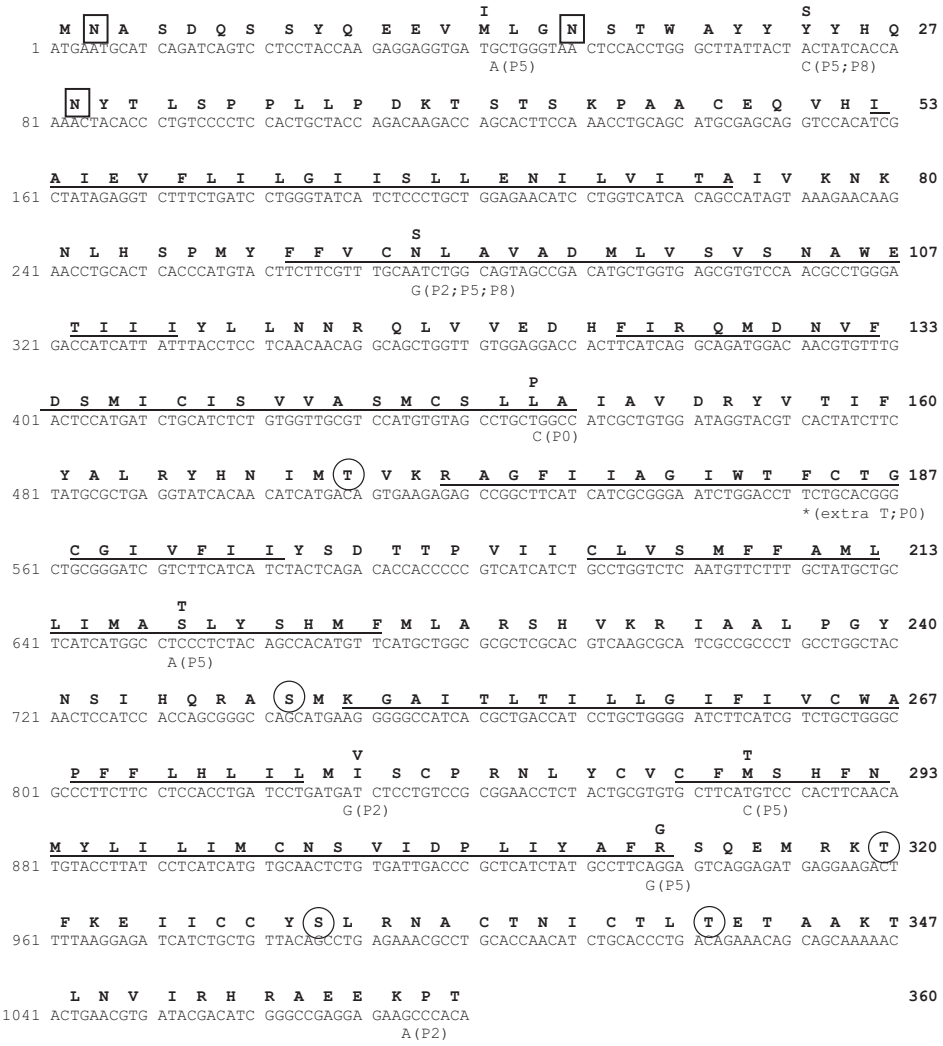

Fig. 1. Nucleotide and deduced amino acid sequence of sea bass melanocortin receptor 5 (sbMC5R), P1 isoform. Nucleotide and amino acid sequence numbers are indicated on the left and right sides, respectively. Position of different polymorphisms and the corresponding isoform are shown. Underlining indicates putative transmembrane domains (Haitina et al., 2007a). Boxes indicate putative *N*-linked glycosylation sites; circles, possible phosphorylation sites. Sea bass MC5R_P1 sequence accession number is FN429769.

(ICL4). The deduced amino acid sequence has potential *N*-glycosylation sites within the N-terminal domain at positions 2, 17 and 28 (Fig. 1). Sea bass MC5R also has a PMY motif in the first ICL, which is conserved in most melanocortin receptors. The motif DRY of TM3, a consensus motif of the class A rodopsin-like G protein-coupled receptor, is also present. Consensus recognition sites for protein kinase C (PKC) and cAMP- and cGMP-dependent protein kinases within ICLs were found at positions 170 in ICL2, 248 in ICL3 and 320, 329 and 339 within ICL3 (Fig. 1), suggesting regulation by phosphorylation.

We found five receptor isoforms (P0, P1, P2, P5, P8), which were sequenced twice on both strands, involving nine single nucleotide polymorphisms (SNPs) at positions 42, 71, 274, 446, 652, 829, 866, 937 and 1074 (Fig. 1). These SNPs result in eight putative amino acid substitutions at positions 14, 24 (ECL1), 92 (TM2), 149 (TM4), 218 (TM6), 277 (ECL4), 289 (TM6) and 313 (ICL4, Fig. 3). Other than P0, P5 was the most divergent isoform, exhibiting four unique substitutions within the ECL1, TMD5, TMD7, ICL4. By contrast, P1 and P2 were found to have unique substitution within TMD2 and ECL4, respectively, whereas P8 had no unique substitutions. There were no coincident substitutions between P2 and P5, different from those observed in P1 and P8. The isoform P0 had an extra thymine base at position 551/552 (Fig. 1), involving a change in the reading frame and resulting in a divergent and shortened C-terminal region of the receptor (Fig. 3). Analysis of the receptor structure predicts the disruption of the transmembrane structure within the C-terminal region.

Peripheral and central distribution of *sbMC5R* mRNA

RT-PCR using specific primers targeting sequences within TMD4 and TMD7 of *sbMC5R* resulted in a band of the expected size of about 436 bp (Fig. 4A). The identity of the band was confirmed by Southern blot hybridization with a *sbMC5R* probe including the full coding region. *sbMC5R* mRNA was easily detected in the retina, brain, liver, spleen, gill, testis and dorsal skin. Low levels were also distinguishable in the pituitary, posterior kidney, fat tissue, intestine, red muscle and ovary (Fig. 3A). Finally residual levels were detected in the head kidney, dorsal skin, white muscle and heart. Inverse transcriptions and cDNA quality were corroborated by PCR amplification of 18S RNA that yielded bands of expected size in all reactions (Fig. 4B).

Activation by melanocortin analogues

For pharmacological and functional characterization of sbMC5R, the coding region of the P5 isoform, was ligated into pcDNA3 and stably expressed in HEK293 cells already producing β-galactosidase under the control of cAMP responsive elements. SbMC5R was positively coupled to the cAMP-signalling pathway in response to all melanocortins tested (Fig. 5). NDP-MSH was the most potent agonist, activating the receptor with a half-maximal effective

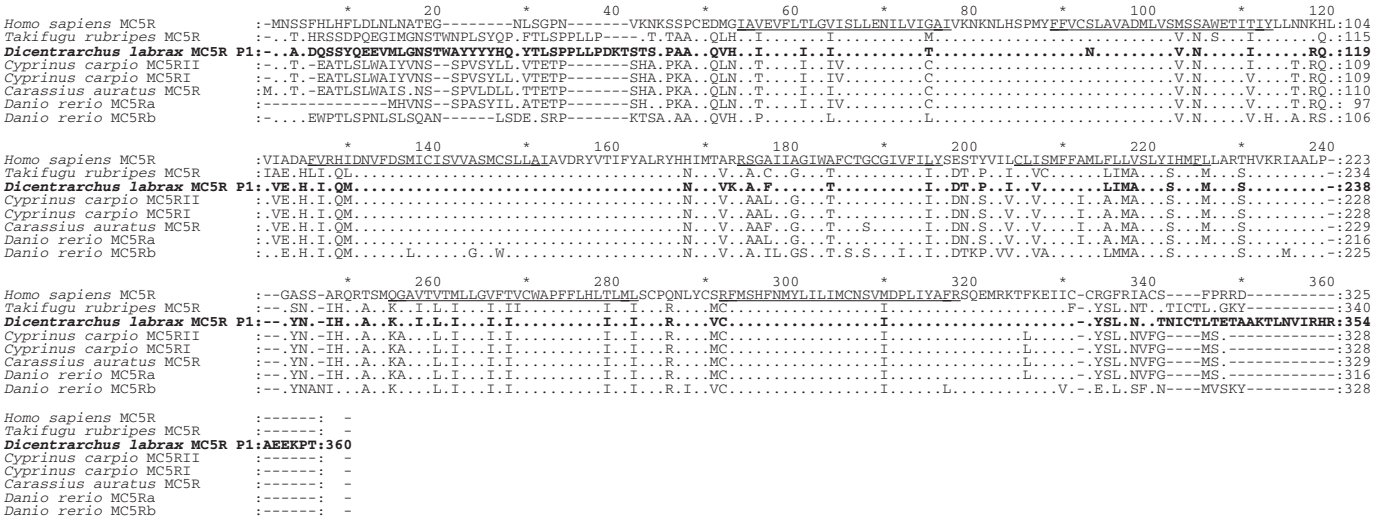

Fig. 2. Alignment of MC5R amino acid sequences. Sea bass MC5R sequence is in bold letters. Dots indicate amino acids identical to the top sequence. Dashes indicate spaces introduced to improve alignment. Grey boxes show putative transmembrane domains (see Fig. 1).

concentration (EC_{50}) of 0.133 nmol l^{-1}. Activation by MTII and SHU9119 showed an EC_{50} of 1.82 nmol l^{-1}, and 3.72 nmol l^{-1}, respectively, whereas the effective concentration increased to 22.4 nmol l^{-1} and 93.3 nmol l^{-1}, and 107 nmol l^{-1} and 167 nmol l^{-1} when cells were incubated with HS024, diacetyl-MSH, α-MSH and monkey β-MSH, respectively. Sigmoid curves cannot be fitted for desacetyl-MSH and ACTH since receptor was only activated by the highest melanocortin concentrations (1 μmol l^{-1}).

SbMC5R was also activated by the non-selective melanocortin agonist MTII (EC_{50}=5.69 nmol l^{-1}). When a phosphodiesterase inhibitor (IBMX) was added to the medium, the response of the reporter gene to incubation with MTII increased (EC_{50}=0.167 nmol l^{-1}). The incubation of sbMC5R-expressing HEK cells with AGRP, in the presence or absence of IBMX, had no effects on receptor activation. Under these conditions, AGRP had no effect on MTII-stimulated cAMP production (EC_{50}=17 nmol l^{-1} and

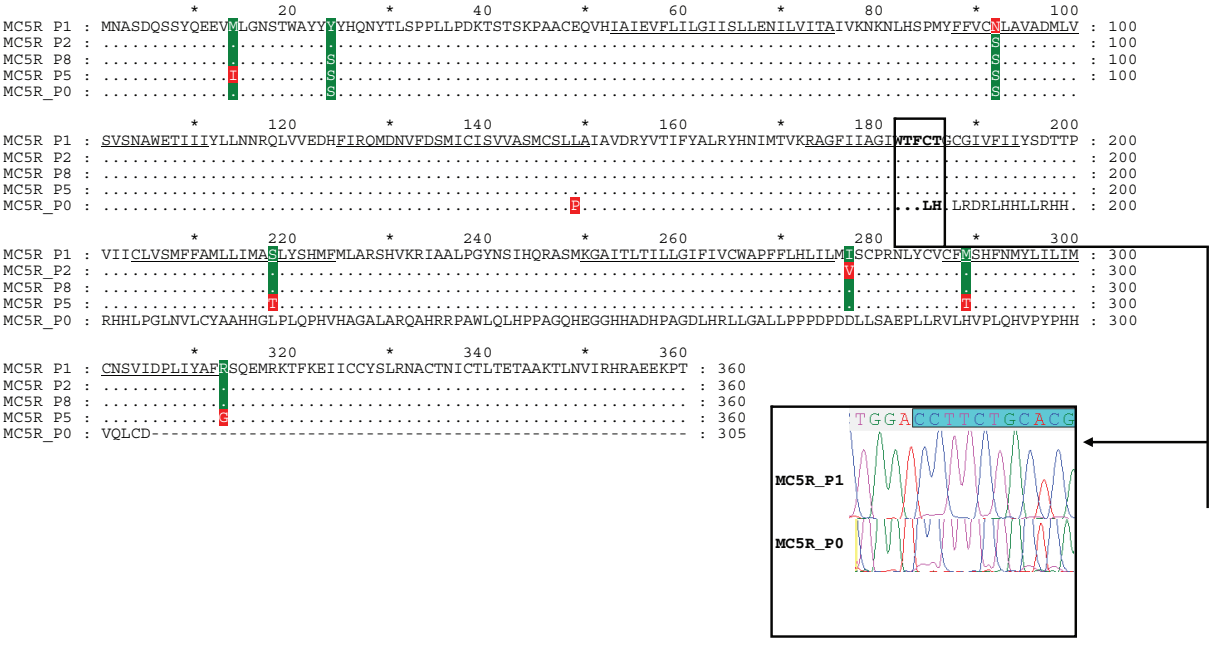

Fig. 3. Alignment of sbMC5R isoforms. Dots indicate amino acids identical to the top sequence. Amino acid sequence numbers are indicated on the right side. Underlined amino acids indicate putative transmembrane domains (see Fig. 1). Amino acids that only change in one sequence are represented in red, whereas amino acids present in at least two sequences are shown in green. Inset shows sequencing chromatograms comparing DNA sequences of isoforms P0 and P1. Note the extra thymine base in the P0 sequence, leading to a shift in the reading frame that results in a shortened and divergent C-terminal region in the P0 isoform. Sea bass MC5R sequence accession numbers: P0: FN429774, P1: FN429769, P2: FN429770, P5: FN429772, P8: FN429773.

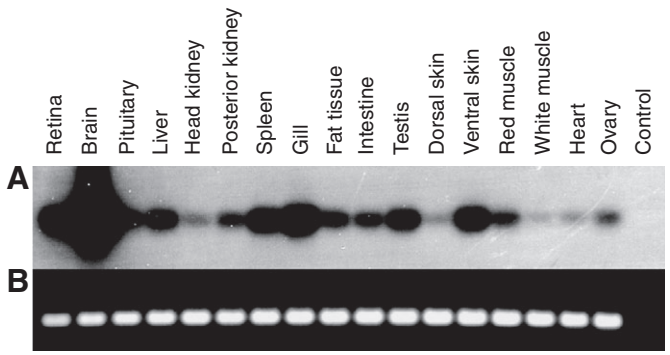

Fig. 4. Distribution of sea bass MCR5 mRNA expression in different tissues, as revealed by RT-PCR followed by Southern blot hybridization. (A) Phosphoimaging screen showing a Southern blot analysis of the sea bass receptor following RT-PCR, and (B) ethidium bromide-stained agarose gel of RT-PCR amplifications of sea bass 18S RNA.

0.15 nmol l^{-1}, in the absence or presence of IBMX, respectively; Fig. 6).

Subsequently, we used HEK293 cells stably expressing a galactosidase reporter gene to transiently transfect the different sbMC5R isoforms (P0, P1, P2, P5, P8). The isoform P0 never responded to any melanocortin peptide, whereas the binding of endogenous melanocortin to the P1-P8 isoforms differentially activated the galactosidase gene transcription. Isoforms P1 and P8 activated the reporter gene more efficiently than P2 and P5 when incubated with ACTH, α-MSH, β-MSH and desacetyl-MSH, but not with diacetyl-MSH (Fig. 7).

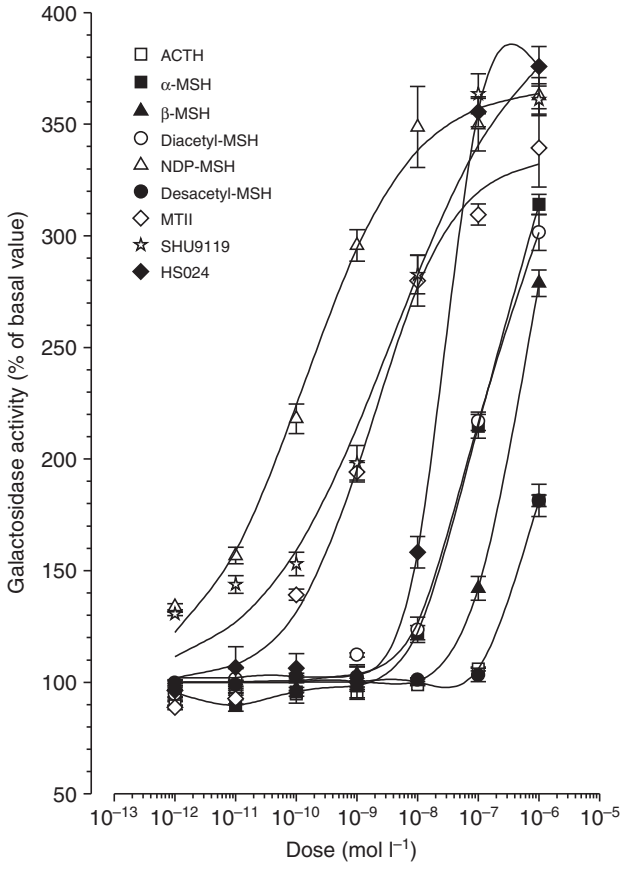

Fig. 5. Effects of synthetic (HS024, MTII, NDP-MSH, SHU9119), and endogenous (ACTH, α-MSH, β-MSH, diacetyl-MSH and desacetyl-MSH) melanocortin on galactosidase activity in HEK293 cells stably expressing both sbMC5R-P5 and a cAMP-responsive β-galactosidase reporter gene in the absence of phosphodiesterase inhibitors (IBMX). Data were normalized to protein levels and expressed as percentage of the basal levels.

Effects of fasting on sbMC5R expression

Quantitative PCR did not yield significant differences in hypothalamic *MC5R* mRNA expression levels when fed and fasted animals were compared after 1, 4, 8, 15 and 29 days of fasting (data not shown).

Effects of melanocortin agonist on hepatic lipolysis

The incubation of liver slices with 10^{-6} mol l^{-1} MTII in HBS resulted in a significant increase in total NEFA concentration in the culture medium after 4 h but not after 2 h (Fig. 8A). Only the highest MTII concentrations significantly stimulated NEFA production *in vitro* (Fig. 8B).

DISCUSSION

The present study provides evidence of the allelic complexity of the *MC5R* locus in sea bass and substantiates the presence of several isoforms that exhibit different sensitivity to endogenous melanocortin agonist. We also describe a non-functional isoform of the sbMC5R with a divergent C-terminal region. Tissue expression patterns revealed high expression levels in the central nervous system with lower levels in several peripheral tissues including liver. The function of MC5R in fish is unknown; however, we demonstrate that MTII, a potent agonist of the sbMC5R,

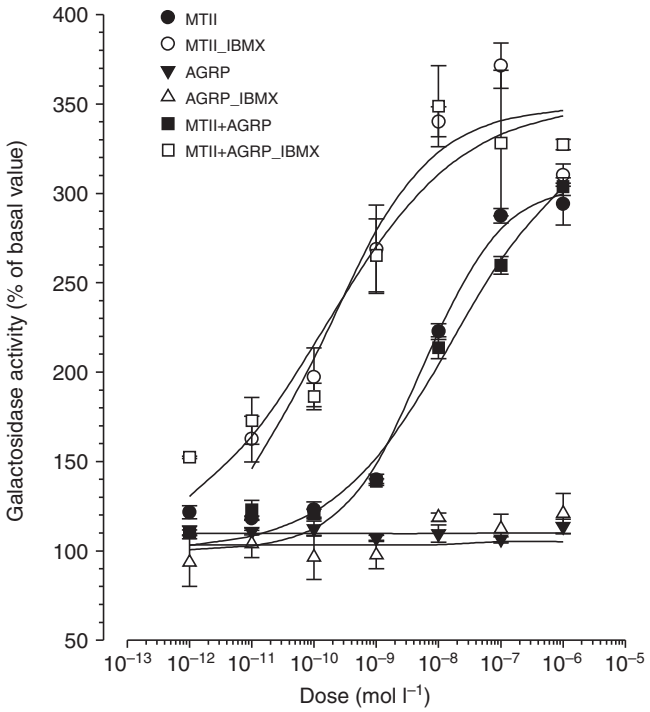

Fig. 6. Effects of inverse agonist, agouti-related protein (AGRP), on basal and MTII-stimulated galactosidase activity in HEK293 cells stably expressing both sbMC5R-P5 and a cAMP-responsive β-galactosidase reporter gene in the presence or absence of phosphodiesterase inhibitors (IBMX). Data were normalized to protein levels and expressed as percentage of the basal levels.

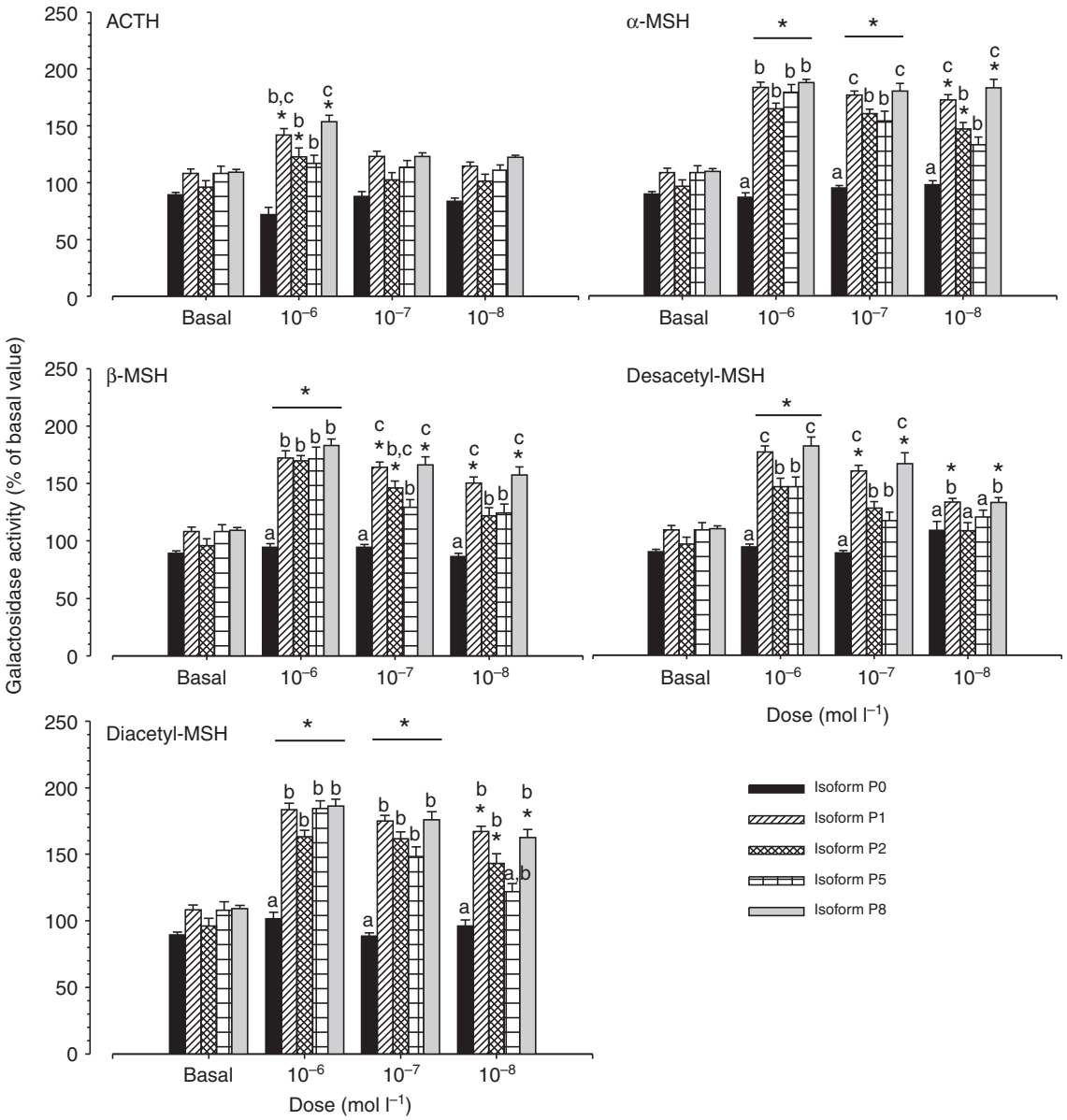

Fig. 7. Effects of endogenous (ACTH, α-MSH, β-MSH, diacetyl-MSH and desacetyl-MSH) melanocortin on galactosidase activity in HEK293 cells transiently expressing different sbMC5R isoforms and the luciferase gene under the control of a constitutive promoter and stably expressing a cAMP-responsive β-galactosidase reporter gene. Experiments were done in the absence of phosphodiesterase inhibitors (IBMX). Data were normalized to protein levels, luciferase activity and forskolin-induced galactosidase activity and expressed as percentage of the common basal levels. Asterisks indicate differences from the basal level of that compound, after one-way ANOVA. Different letters indicate differences between isoforms at the specified hormone concentration, after one-way ANOVA (P<0.05).

stimulates hepatic lipolysis, suggesting that the different MC5R isoforms could play a role in hepatic metabolism.

Amino acid sequence comparisons show that the cloned receptor in sea bass displays high identity with other cloned MC5Rs. Most of the divergence in sbMC5R is within the N-terminal extracellular domain. However, this receptor domain is not likely to be functionally important for agonist binding, since it has been demonstrated that 28 amino acids from the N-terminal extracellular domain can be deleted, including all potential *N*-glycosylation sites, without affecting ligand binding to human MC5R (Schiöth et al., 1997). Similarly, ECLs of the mouse MC5R do not participate in the ligand binding of melanocortin agonist (Schiöth et al., 1998). Accordingly, sbMC5R has low sequence identity with other MC5Rs within the ECLs but, in contrast, shares a high sequence identity with other MC5Rs within the TMDs. Point mutagenesis of human MC1R suggests that important determinants for agonist binding lie within TMD2, TMD3 and TMD7 (Yang et al., 1997), where sbMC5R has higher than 82%, 83% and 92% sequence identity, respectively, when compared with other MC5Rs.

To our knowledge, the *MC5R* locus, unlike *MC1R,* has not been reported to be highly polymorphic in species in which the receptor has been cloned (García-Borrón et al., 2005; Sanchez et al., 2009b). However, we found nine DNA polymorphisms during the cloning process. Eight mutations resulted in substitution of a different amino acid and only one was a silent mutation. These polymorphisms were incorporated into five different isoforms. Furthermore the P0

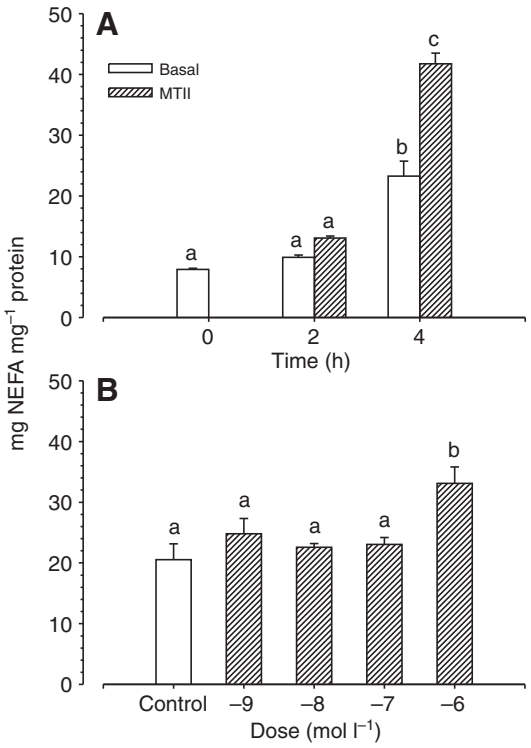

Fig. 8. Effects of melanocortin agonist MTII on hepatic lipolysis measured as release of non-esterified fatty acids (NEFA) after hormonal treatment of the culture medium. (A) Time course experiments using MTII at 10^{-6} mol l^{-1} after 2 and 4 h incubation. (B) Dose–response assay after 4 h incubation.

isoform had an extra thymine that induces a change in the reading frame, resulting in a divergent and shortened C-terminal region. We initially selected the P5 isoform for pharmacological characterization of the sbMC5R. Activation experiments demonstrated that sbMC5R is activated by all melanocortin mimetics. NDP-MSH, MTII and SHU9119, antagonists of sbMC4R (Sánchez et al., 2009a), were the most potent agonists of the sbMC5R with ED_{50}s within the nanomolar range. Similarly, NDP-MSH was also the most potent agonist of zebrafish (Ringholm et al., 2002) and pufferfish MC5Rs (Klovins et al., 2004). Both diacetyl-MSH and α-MSH similarly stimulated cAMP-dependent galactosidase activity but desacetyl-MSH-induced activation was less efficient. Therefore, acetylation seems to be critical for sbMC5R activation but not the level of acetylation since monoacetyl (α-MSH) and diacetyl-MSH were equipotent when activating the receptor. All three MSH isoforms have been described in fish pituitary (Dores et al., 1993) and the regulation of MSH acetylation seems to be an important mechanism for background adaptation and pigment patterns (Arends et al., 2000; Kobayashi et al., 2009). SbMC5R was weakly activated by incubation with human ACTH and β-MSH. Experiments in trout demonstrated that MC5R affinity for human ACTH (1-24) was higher than that observed for α-MSH and β-MSH, both exhibiting equipotent binding capacities on trout MC5R (Haitina et al., 2004). Our data suggest that sbMC5R is not specific for ACTH or β-MSH, although no definitive conclusions can be drawn since we used human and monkey peptides, respectively. Studies in lamprey have also demonstrated that the cloned MCR subtype exhibits higher affinity to the lamprey ACTH than to the MSH-A suggesting that ACTH is the ancestral ligand (Haitina et al., 2007b).

Previous experiments in our laboratory demonstrated that zebrafish AGRP (zfAGRP) is able to decrease the basal activity of both sbMC4R and sbMC1R when incubated with a phosphodiesterase inhibitor (IBMX), as occurs with an inverse agonist, and decrease the effects of the melanocortin agonists on the sbMC4R activation (Sánchez et al., 2009a; Sánchez et al., 2009b), as occurs with an endogenous antagonist. We further demonstrate here that this effect is specific for sbMC1R and sbMC4R, since zfAGRP had no effect on basal sbMC5R activity in the absence or presence of phosphodiesterase inhibitors. In addition, our data corroborate the role of the phosphodiesterase system in the AGRP inverse agonism. As expected, the addition of IBMX decreased ED_{50} values of agonist-induced galactosidase activity, thus increasing the sensitivity of the assay. In addition, AGRP could not decrease the MTII-induced activation of the sbMC5R as a competitive antagonist does. When we further studied the activation of the different sbMC5R isoforms by the endogenous melanocortin agonist, the P0 isoform, which has a shortened and highly divergent C-terminal region, was ineffective in mediating the cAMP-induced galactosidase activity upon exposure to natural ligands. However, from our data it is not possible to discriminate whether the binding properties of the receptor or its coupling to G-proteins were disrupted. However, they strongly suggest that P0 is a non-functional receptor isoform. The P1 and P8 isoforms displayed higher sensitivity to endogenous melanocortins than the P2 and P5 isoforms, suggesting that specific MC5R allelic combinations may confer differential sensitivity to endogenous melanocortin. It is difficult to derive any conclusions concerning any structure–activity relationship among active isoforms since no common substitution patterns between P2/P5 and P1/P8 were found.

MC5R has different expression patterns in different vertebrate lineages. As in mammalian species, sbMC5R was expressed in a variety of peripheral tissues (Chen et al., 1997). Although no quantitative experiments were carried out, *sbMC5R* mRNA levels in the CNS seem to be higher than in the peripheral tissues. High expression levels in the brain were also reported in other fish species including zebrafish (Ringholm et al., 2002), goldfish (Cerdá-Reverter et al., 2003b), pufferfish (Klovins et al., 2004), trout (Haitina et al., 2004) and carp (Metz et al., 2005). The CNS expression of MC5R has also been reported in human (Chhajlani et al., 1993), rat (Griffon et al., 1994) and chicken (Takeuchi and Takahashi, 1998) but very low levels were detected in mouse brain (Gantz et al., 1994). The central function of MC5R is unknown but experiments in rodent have demonstrated that central administration of melanocortin stimulates aggression. By contrast, MC5R-deficient mice exhibited reduced aggression and an elevated defence against wild-type opponents (Morgan and Cone, 2006). The high expression levels of MC5R in fish brain suggest implemented central functions compared with mammalian systems. Our previous experiments in goldfish demonstrated that the melanocortin system is involved in the control of food intake. Central administration of melanocortin analogues inhibit food intake (Cerdá-Reverter et al., 2003a; Cerdá-Reverter et al., 2003b), whereas fasting dramatically increases hypothalamic AGRP expression (Cerdá-Reverter and Peter, 2003). Similar to sbMC4R (Sánchez et al., 2009a), progressive fasting did not induce changes in hypothalamic sbMC5R expression, suggesting that food deprivation cannot induce a downregulation of receptor levels in the hypothalamic structures. The sbMC4R exhibits constitutive activity and its actions are regulated by binding of the inverse agonist AGRP (Sánchez et al., 2009a). The above model cannot account for the MC5R-regulated hypothalamic pathways since AGRP does not work as an inverse agonist or competitive

antagonist. POMC expression levels in the brain were also constant after progressive fasting in sea bass (Sánchez et al., 2009a) and goldfish (Cerdá-Reverter et al., 2003c). In the absence of modulation of POMC expression levels by fasting and of any effect of AGRP on basal or stimulated sbMC5R activity, an up- or downregulation of sbMC5R expression after fasting would seem likely if the receptor is involved in the control of food intake. Therefore, our data do not support the involvement of this receptor in the control of food intake in fish.

In mice, MC5R deficiency also causes severe dysfunction of the exocrine glands by mis-regulation of sebaceous and preputial lipid synthesis (Chen et al., 1997). We and others have found high levels of *MC5R* mRNA in the fish skin and gills (Cerdá-Reverter et al., 2003b; Metz et al., 2005) (present results), suggesting that MC5R may be involved in the mucus production by globet cells present in the gills and skin, and/or additional secretory skin cells (Shepard, 1994). RT-PCR experiments demonstrated expression of sbMC5R in the ventral skin but no expression was found in the dorsal region. Similar to many other vertebrates, sea bass have a specific pigment pattern with darker colour in the dorsal area and a progressive gradation towards white-yellow tones in the abdominal skin. Differential MC5R expression in the ventral skin suggests a role in pigment synthesis of the sea bass. Interestingly, sbMC5R was expressed in tissues containing a high number of macrophages, i.e. spleen, head kidney and gills, thus suggesting a role in the immune system. Accordingly α-MSH has been demonstrated to modulate fish immune system (Harris and Bird, 2000). Moderate levels of expression were also found in the liver and adipose tissue. Involvement of the melanocortin system in the control of the fat balance of fish is corroborated by the 'cobalt' phenotype of rainbow trout. The absence of most of the pars intermedia of the pituitary, where α-MSH is synthesized, seems to be responsible for this phenotype. This 'cobalt' trout is hyperphagic and exhibits an enlarged liver and fat accumulation in the abdominal cavity, reflecting the absence of α-MSH lipolytic activity. This suggests that the melanocortin system plays a role in the hepatic metabolism by stimulating peripheral lipolytic activity, thus enhancing energy expenditure. Our results further demonstrate that MTII, an agonist of sbMC5R, is able to stimulate hepatic lipolysis *in vitro*, measured as free fatty acid release into the culture medium after melanocortin agonist exposure of liver fragments. This suggests that different allelic composition results in differential hepatic sensibility to fasting in sea bass. However, our experiments cannot fully exclude the participation of other melanocortin receptors expressed in the sea bass liver. Previous experiments have demonstrated the presence of residual levels of MC1R and MC4R in the sea bass liver (Sánchez et al., 2009a; Sánchez et al., 2009b). Our unpublished experiments also demonstrated the expression of MC2R in the sea bass liver but this receptor is specific for ACTH peptides in sea bass (M. J. Agulleiro, E.S., V.C.R. and J.M.C.-R., unpublished data).

In summary, we report the molecular and pharmacological characterization of sbMC5R, and point to its potential participation in hepatic metabolism by promotion of lipolysis. *sbMC5R* is a complex locus with at least five different alleles, resulting in five isoforms. One of these isoforms was non-functional and the remaining four exhibited different degrees of sensitivity to melanocortin peptides. The results suggest that the different allelic composition of the *sbMC5R* locus might result in different phenotypes exhibiting differential activity in the hepatic metabolism.

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