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Gene Structure of the Goldfish Agouti-Signaling Protein: A Putative Role in the Dorsal-Ventral Pigment Pattern of Fish

José Miguel Cerdá-Reverter, Tatjana Haitina, Helgi Birgir Schiöth, and Richard Ector Peter

Department of Fish Reproductive Physiology (J.M.C.-R.), Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Cientificas, Castellón 12595, Spain; Department of Neuroscience (T.H., H.B.S.), Unit of Pharmacology, Uppsala University, SE 751 24 Uppsala, Sweden; and Department of Biological Sciences (J.M.C.-R., R.E.P.), University of Alberta, Edmonton, Alberta, Canada T6G 2E9

One of the most successful chromatic adaptations in vertebrates is the dorsal-ventral pigment pattern in which the dorsal skin is darkly colored, whereas the ventrum is light. In fish, the latter pattern is achieved because a melanization inhibition factor inhibits melanoblast differentiation and supports iridophore proliferation in the ventrum. In rodents, the patterned pigmentation results from regional production of the agouti-signaling protein (ASP). This peptide controls the switch between production of eumelanin and pheomelanin by antagonizing αMSH effects on melanocortin receptor (MCR) 1 in the melanocytes. In addition, ASP inhibits the differentiation and proliferation of melanoblast. Thus, the mammalian ASP may be homologous to the poikilotherm melanization inhibition factor. By screening of a genomic library, we deduced the amino acid sequence of goldfish ASP. The ASP gene is a four-exon gene spanning 3097 bp that encodes a 125-amino acid precursor. Northern blot analysis identified two different ASP mRNAs in ventral skin of red- and black-pigmented and albino fish, but no expression levels were observed in the dorsal skin of the same fish. The dorsal-ventral expression polarity was also detected in both black dorsally pigmented fish and albino fish. Pharmacological studies demonstrate that goldfish ASP acts as a melanocortin antagonist at *Fugu* MC1R and goldfish MC4R. In addition, goldfish ASP inhibited Nle4, D-Phe7-MSH-stimulated pigment dispersion in medaka melanophores. Our studies support agouti signaling protein as the melanization inhibition factor, a key factor in the development of the dorsal-ventral pigment pattern in fish. (*Endocrinology* 146: 1597–1610, 2005)

IN TELEOSTS, PIGMENT cells are commonly found in the dermis and can be divided into light-absorbing (melanophores, xantophores, erythrophores, and cyanophores) and light-reflecting (leucophores and iridophores) chromatophores (1). Fish melanophores are known to contain only eumelanins (black-brown pigments), whereas xantophores and erytrophores contain carotenoids and/or pteridines that contribute to reddish and yellowish components of the skin coloration. Iridophores are commonly localized in whitish and silvery areas of the skin, predominantly in the belly surface. They contain crystalline platelets composed of purines, mainly of guanine, responsible for the reflection of the light (2). The light interaction with patterned distribution of the pigmented cells creates the color pattern in fish. Although this pigment pattern is most evident in the adult animal, their cellular basis is established during embryogenesis (3–6).

One of the most successful chromatic adaptations in vertebrates including fish is the dorsal-ventral pigment pattern in which the dorsal skin is darkly colored, whereas the ventrum is light.

In teleosts, this countershading is achieved by a patterned distribution of the pigment cells, with the light-absorbing and light reflecting chromatophores mostly distributed in the dorsal and ventral areas, respectively (7, 8). Experimental data in amphibian and fish species support that the dorsal-ventral pigment pattern is achieved because a putative diffusible melanization inhibition factor (MIF), mainly produced by cells in the ventral skin, inhibits melanoblast differentiation and stimulates or supports iridophore proliferation in the ventrum (7, 9, 10). Amphibian MIF has been reported to block the stimulation of melanization provoked by αMSH in neural explants of *Xenopus* (11). Similarly, amphibian MIF has been shown to block αMSH-induced tyrosine hydroxylase and dopa oxydase activity in mouse malignant melanocytes; both key enzymes in the melanogenic pathway (12). Although MIF has been partially purified and MIF antibodies have localized its expression in the ventral skin of leopard frog (*Rana forreri*), the molecular structure remains as yet unidentified (13).

In mice, the difference in dorsal and ventral pigmentation results from local production of the agouti protein (14), designated as agouti-signaling protein (ASP) in species different from mouse. Agouti gene encodes a 131-amino acid protein with structural characteristics of a secreted protein having a hydrophobic signal sequence and lacking any transmembrane domain (15). A highly basic domain with a high pro-

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Abbreviations: AGRP, Agouti-related protein; ASP, agouti-signaling protein; HEK, human embryonic kidney; MCR, melanocortin receptor; MIF, melanization inhibition factor; MIFT, αMSH-induced expression of microphthalmia; NDP-MSH, (Nle4, D-Phe7)-αMSH; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SSPE, sodium chloride/sodium phosphate/EDTA; UTR, untranslated region.

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portion of arginine and lysine residues forms the N-terminal region of the agouti protein. The latter region leads a proline-rich area that immediately precedes the cysteine rich C-terminal domain. This cysteine domain resembles the conotoxins and plectoxins of snails and spiders, respectively (16). Agouti is mainly produced by dermal papillae cells controlling the switch between production of eumelanin (black/brown pigment) and pheomelanin (yellow/red pigment) by antagonizing αMSH effects on melanocortin receptor (MCR) 1 in the follicle melanocytes (17). In addition, agouti has been also demonstrated to inhibit the differentiation and proliferation of melanoblasts (18, 19). The unusual allele of the agouti locus, A^y, consists of a large deletion in the noncoding regions that places agouti gene under the control of the *Raly* promoter, a ubiquitously expressed RNA binding protein. The associated phenotype 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 (20, 21). This metabolic syndrome is mediated by antagonizing αMSH signaling at central MC4R that arbitrate negative effects of melanocortin peptides on energy balance (22). The ectopic expression of agouti in the brain mimics the action of agouti-related protein (AGRP), a central antagonist that regulates the inhibitory tone imposed by melanocortins on food intake at MC3R and MC4R (23). AGRP lacks the highly basic N-terminal and proline-rich regions, but it shares strong homology to agouti protein within the poly cysteine domain (24, 25). The latter protein has been characterized in several fish species including goldfish (26–28).

Although it is difficult to compare fish scales with mammalian hairs, the molecular mechanisms involved in establishing pigment patterns are conserved (3, 4, 29). Because amphibian MIF seems to interact with the melanocortin system by blocking αMSH effects (11, 12), mammalian ASP may be homologous to the poikilotherm MIF, but ASP has never been identified in any nonmammalian species. The aim of this work was to explore the possible existence of ASP in fish as well as its relationship to the dorsal-ventral pigment pattern.

Materials and Methods

Animals and reagents

Male and female common goldfish (*Carassius auratus*) were purchased from Mount Parnell Fisheries (Mercersburg, PA). Albino and black dorsally pigmented goldfish were mixed among the red fish provided by the supplier. Fish were fed a 2% body weight ration once a day with trout pellets (Moore Clark, St. Andrews, New Brunswick, Canada). Medaka broodstock (SOK strain) was kindly provided by Dr. M. Schartl (University of Wurzburg, Wurzburg, Germany). Animals were anesthetized in 0.02% tricaine methasulfonate (MS-222, Syndel Laboratories Vancouver, British Columbia, Canada) for 2 min before any manipulation. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals. Unless otherwise indicated, all reagents were purchased from Sigma (St. Louis, MO).

Molecular cloning, gene structure, and DNA sequencing

Filters from a goldfish genomic library made in the vector λ-GEM-11 (kindly provided by Dr. K. L Yu, Department of Zoology, University of Hong Kong, Hong Kong), containing approximately 10^6 clones, were screened with a mouse AGRP probe consisting of the exon 4 (kindly provided by Dr. G. S. Barsh, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA). Membranes were hybridized in hybridization solution [35% formamide, 6× sodium chloride/sodium phosphate/EDTA (SSPE), 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, 10 μg/ml yeast tRNA type III, 1× SSPE containing 150 mM NaCl, 1 mM EDTA, and 9 mM NaH_2PO_4 (pH 7.4)] containing 6× 10^5 cpm/ml dCTP α-^{32}P at 42 C. Final washes were performed in 1× SSPE at 42 C. Two clones named 1.1.1.1 and 5.2.4.1 were isolated. A fragment expanding 3.2 kb from clone 1.1.1.1 was sequenced on both strands by using an automated sequencer (373A) (Applied Biosystems, Foster City, CA) and found to contain a translated region showing a high homology level to mouse agouti exon 4. The clone 5.2.4.1 was found to encode goldfish AGRP (26). After analysis of the possible translations of the 1.1.1.1 clone, a putative signal peptide was detected 937 bp downstream of the 5′ end. RT-PCR using ventral skin cDNA as template and AgSPFw/AgR2 primers generated a 375-bp fragment. The primer AgSPFw, designed to target the putative signal peptide, had the sequence 5′-ATC CGT CAT TGT TGC TGT G-3′. The primer AgR2 had the sequence primer (5′-GTT ACC CGA AAT GCT GAA-3′). The fragment was isolated, subcloned into pGEM-T Easy Vector System (Promega, Madison, WI), and sequenced as before. Two additional low intensity 592- and 532-bp bands were detected when amplifying ventral skin cDNA with AgSPFw/AgR2 or AgFwDTC/AgR2 primers, respectively. The 532-bp fragment from the amplification AgFwDTC/AgR2 was cloned as above, and two slightly different size clones were sequenced and aligned to the genomic sequence. AgFwDTC primer had the sequence 5′-ACA CAT GAT TAT AGA GGA ACA A-3′.

To resolve both 5′ and 3′ ends of goldfish ASP cDNA, 5′ and 3′ rapid amplification of cDNA ends (RACE)-PCR was performed as described earlier (30). For 3′ RACE-PCR, cDNA from ventral skin mRNA was synthesized using dT-adapter primer (5′-GGC CAC GCG TCG ACT AGT AC$(T)_{17}$-3′). Two rounds of PCR amplified the 3′ end with adapter (5′-GGC CAC GCG TCG ACT AGT AC-3′) and AgFwDTC primers and then adapter primer and AgE4Fw primers (5′-ACC CAT GTG AAG AGA CCT-3′). After low-melting-point purification, a 981-bp fragment was subcloned into pGEM-T easy vector and two clones were sequenced. For 5′ RACE-PCR, cDNA synthesis from ventral skin mRNA was primed with AgR2, and cDNAs were polyadenylated at 3′ end using terminal dideoxy transferase (Promega). The polyadenylated cDNAs were then amplified by two rounds of PCR first using dT-JAP-adapter (5′-CAG TCG AGT CGA CAT CGA $(T)_{12}$-3′), JAP-adapter (5′-CAG TCG AGT CGA CAT CGA-3′), and AgRevDTC primer (5′-GAA CAG GCG GCA GTG GC-3′). Nested PCR was done by using JAP-adapter and 5Ag2 primer (5′-CGA CTA TGA GCA CAG AAG G-3′). Three different 255-, 286-,and 379-bp bands were cloned into pGEM-T easy vector and sequenced as above. Finally, ventral skin cDNA was amplified with primers AgFullFw (5′-ATC TCA AGC CAG CTA CAC-3′) and AgFullRev (5′-GAT TCA AGA GCA ATG GAT G-3′) and two 1263- and 1140-bp bands were cloned and sequenced. Primers used for the different amplifications are shown in Fig. 1.

RT-PCR and Southern blot analysis

Total RNA was purified with Trizol (Invitrogen, Carlsbad, CA) and treated with RQ1-DNase (Promega). Superscript II reverse transcriptase (Invitrogen) was used for cDNA synthesis by priming total RNA from testis, ovary, brain, pituitary, eye, gill, muscle, fat, skin, spleen, kidney, intestine, heart, and liver with oligo $(dT)_{12–18}$ (Invitrogen). PCR amplification was carried out with the primers AgFwDTC and AgR2, thus predicting a 314-bp amplification band. Subsequently, PCR fragments were separated onto 1.2% agarose gel and transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL). Hybridizations were carried out in hybridization solution (50% formamide, 6× SSPE, 0.5% SDS, 5× Denhardt's solution, and 10 μg/ml yeast tRNA type III) using full coding region cDNA as probe. Final washes were performed in 0.1× SSPE at 65 C.

To study the dorsal-ventral polarity of agouti expression, mRNA from dorsal or ventral skin of red dorsally pigmented fish was isolated using the polyATrack mRNA isolation system III (Promega) following the manufacturer's manual. cDNAs were synthesized by priming 0.5 μg mRNA with dT-adapter and purified using Centricon columns (Milli-

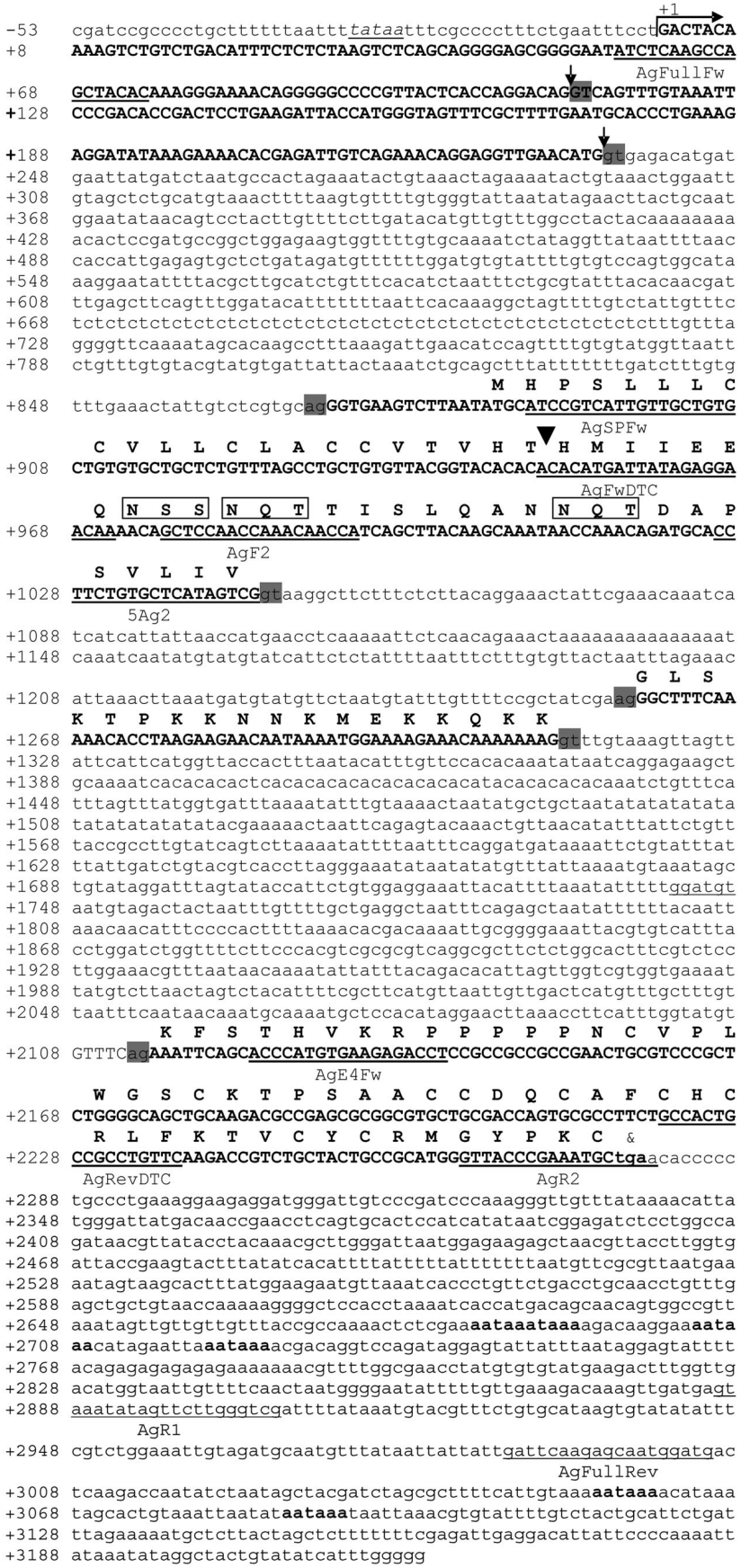

FIG. 1. Nucleotide and deduced amino acid sequence of the goldfish ASP gene. Nucleotide numbers are indicated on the *left*. Introns are shown in *lower-case letters*, whereas exons are shown in *bold upper-case letters*. *Arrows* indicate positions for the alternative splicing of exon 1. *Gray boxes* frame exon-intron splice junctions. The stop codon is marked (&), and nucleotides of the consensus polyadenylation site (aataaa) are shown in *bold lower-case letters*. *Arrowhead* indicates the predicted cleavage site for removal of the hydrophobic signal peptide. *Open boxes* frame tripeptide sequences that conform to the consensus sequence for *N*-linked glycosylation sites. Nucleotide sequences of primers used in 5′, 3′, and RT-PCR amplifications are *underlined* (EMBL nucleotide sequence database accession no. AJ845197).

pore, Ibérica, Spain). Elution volume was made up to 100 μl with diethylpyrocarbonate-treated water, and 1 μl of ventral or dorsal cDNA was used for PCR amplification with either AgFWDTC-AgR1 or AgFWDTC-AgR2 primer sets.

Northern blot

Total RNA from dorsal and ventral skin from red and black dorsally pigmented, albino fish, and transfected human embryonic kidney (HEK)-293 cells (see below) was extracted with Trizol and its concentration estimated based on absorbance at 260 nm. About 15 μg total RNA from dorsal and ventral skin, or 10 μg from HEK-293 transfected cells, were electrophoresed onto 1.5% agarose gel containing 2.2 M formaldehyde and transferred by capillarity to Hybond-N nylon membranes (Amersham). Membranes were prehybridized for at least 3 h in hybridization solution (50% formamide, 5× SSPE, 1% SDS, 5× Denhardt's solution, and 20 μg/ml yeast tRNA type III). Full coding region ASP was used as probe. Hybridization was carried out overnight in fresh hybridization solution containing 2× 10^6 cpm/ ml dCTP [α-^{32}P] at 42 C. Final washes were performed in 0.1× SSPE/0.1% SDS at 65 C.

Goldfish ASP-conditioned medium

The entire coding region of the goldfish ASP sequence was amplified by PCR using Vent DNA polymerase (New England Biolabs Inc., Beverly, MA) and clone AGSP as template. The so-called AGSP clone displayed two nucleotide substitutions that bring about an M/T amino acid substitution at position 19, inside the putative signal peptide (see *Results*). Specific primers were used under the following conditions: 5 min at 95 C for one cycle, 30 sec at 95 C, and 30 sec at 52–47 C, decreasing 0.5 C for 10 cycles and 20 sec at 72 C, and then 30 sec at 95 C, 30 sec at 47 C, and 30 sec at 72 C for 20 cycles followed by a final cycle at 72 C for 10 min (0.2 mM deoxynucleotide triphosphate, 0.4 μM AG-FOR, and AG-REV primers, 1× *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, and 0.5 U Vent DNA polymerase). The 5′ primer (AG-FOR) was an 18-mer with the sequence 5′-ATG CAT CCG TCA TTG TTG-3′. The 3′ primer (AG-REV) had the sequence 5′-TCA GCA TTT CGG GTA AC-3′. PCR fragments were isolated from low melting point gel and ligated into a pPCR-Script SK (Stratagene, La Jolla, CA). The right orientation was checked by PCR, and subsequently goldfish ASP sequence was released by double digestion with *Not*I/*Bam*HI (Invitrogen) and directionally subcloned into pcDNA3 expression vector (Invitrogen). PcDNA3 inserts were sequenced entirely and found to be identical with the goldfish AGSP clone. The new construct (20 μg) or intact pcDNA3 (20 μg) was transiently transfected in the HEK-293 cell line by using calcium chloride method (31). Cells were grown in DMEM supplemented with 862 mg/liter GlutaMAX I, 4500 mg/liter D-glucose, 110 mg/liter sodium pyruvate (Invitrogen), 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were kept in a humidified atmosphere of 95% air-5% CO_2 at 37 C. The day before the transfection, cells in two 100-mm culture dishes grown at 100% confluence were split in six 100-mm dishes. Three dishes were transfected with intact pcDNA3, and the three others with the new construct carrying goldfish AGSP cDNA. Culture medium was collected at 24, 48, and 78 h post transfection and centrifuged to remove residual cells. Supernatant was stored at −80 C until use. After having removed the medium, cells were washed twice with PBS, and harvested for RNA extraction (see above).

Radioligand binding assay

HEK-293 cell lines expressing *Fugu* melanocortin 1 and goldfish MC4Rs were obtained from the previous studies (28, 31). The cells were grown in DMEM/F-12 nutrient mixture (1:1) with GlutaMAX I containing 10% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 250 μg/ml geneticin G-418 (Life Technologies, Inc., Sweden), and 100 μg/ml hygromycin B (Invitrogen, Sweden); in humidified atmosphere of 95% air-5% CO_2 at 37 C. The cells were harvested from plates and resuspended in binding buffer composed of 25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2% bacitracin (pH adjusted to 7.4). To obtain the membranes, cells were homogenized with a mechanical homogenizator (Ultra Turrax, Tamro, Sweden). The cell suspension was centrifuged for 3 min at 1,300 rpm, and membranes were collected from the supernatant by centrifugation for 15 min at 14,000 rpm. The pellet was resuspended in binding buffer. The binding was performed in a final volume of 100 μl for 3 h at room temperature. Competition experiments were performed with constant 0.4 nM concentration of [^{125}I] (Nle4, D-Phe7)-αMSH (NDP-MSH), labeled by the chloramine-T method (32). Unlabeled NDP-MSH (Neosystem, Strasbourg, France) was used as competing ligand in serial dilutions 1:3, starting from 1 μM concentration. Agouti, present in the medium harvested 48 h after transfection, was also used as competing ligand. Starting from the maximal amount of 50 μl, the medium was serially diluted 1:2 in 12 points. The membranes were collected by filtration on glass fiber filters (Filtermat A, Wallac, Sweden), using a TOMTEC Mach III cell harvester (Orange, CT). The filters were washed with 5 ml/well of 50 mM Tris HCl (pH 7.4) and dried at 50 C. MeltiLex A scintillator sheets (Wallac) were melted on dried filters, and radioactivity was counted with an automatic Microbeta counter 1450 (Wallac). Binding assays were performed in duplicate from at least three independent experiments. Conditioned medium from nontransfected cells or cells transfected with pcDNA vector did not show any specific binding to tested MCRs.

cAMP assay

cAMP production was determined on HEK-293 cells expressing *Fugu* MC1 and goldfish MC4Rs (28, 33). A confluent layer of cells was incubated for 3 h with 2.5 μCi/ml [^{3}H]ATP (specific activity 29 Ci/mmol; Amersham Biosciences, Uppsala, Sweden). Cells were collected and washed with buffer containing 137 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2 \times 6H_2O$, 20 mM HEPES, 1 mM $CaCl_2$, and 10 mM glucose (pH adjusted to 7.4). All reaction components were resuspended in above-mentioned buffer containing 0.5 mM isobutylmethylxanthine (Sigma, Mannheim, Germany). Resuspended cells were incubated for 10 min at 37 C. Stimulation reaction was performed for 20 min at 37 C in a final volume of 150 μl containing approximately 2 × 10^5 cells and serial dilution of αMSH alone or with a fixed amount of 40 μl of medium containing ASP, harvested 48 h after transfection.

After incubation cells were centrifuged and 200 μl of 0.33 M perchloric acid were added to pellets to lyse the cells. The cells were frozen, thawed, and collected at the bottom of the wells by centrifugation at 2000 rpm for 10 min. Two hundred microliters of lysate were added to Dowex 50W-X4 resin columns (Bio-Rad Laboratories, Hercules, CA), previously washed with 2 × 10 ml H_2O. As an internal standard, 750 μl of 0.33 M perchloric acid containing 0.5 nCi/ml [^{14}C]cAMP (Amersham Biosciences) was added to each column. Columns were washed with 2 ml H_2O to remove ATP, which was collected in scintillation vials to estimate the amount of unconverted [^{3}H]ATP. Four milliliters of Ready Safe scintillation cocktail (PerkinElmer, Norwalk, CT) were added to the vials before counting. Dowex columns were then placed over alumina (Sigma) columns (prewashed with 8 ml 0.1 M imidazole), and the cAMP was transferred onto the alumina columns using 10 ml H_2O. The cAMP was eluted from alumina columns with 4 ml 0.1 M imidazole and collected into scintillation vials to which 7 ml of scintillation fluid were added. ^{3}H and ^{14}C were counted in a Tri-carb liquid scintillation β-counter. The amount of obtained [^{14}C]cAMP was expressed as a fraction of total [^{14}C]cAMP ([^{14}C]cAMP/total [^{14}C]cAMP) and was used to normalize the amounts of [^{3}H]cAMP between the columns. Results were calculated as the percent of total [^{3}H]ATP (obtained as a sum of [^{3}H]ATP from first column and [^{3}H]cAMP from second column) to [^{3}H]cAMP and used to determine EC_{50} values by nonlinear regression using Prism software (version 3.0, GraphPad Inc., San Diego, CA). All experiments were performed in duplicate and repeated three times.

Medaka scale bioassay

Adult medaka were acclimated for at least 5 d to a black background. Denervation of melanophores was performed by using reserpine, which is known to deplete adrenergic neurotransmitters in sympathetic postganglionic neurons. Reserpine was diluted in chloroform and subsequently in brackish water (salinity 4‰) to reach a final concentration of 3 μM (34). After 24-h reserpine treatment, the scales from the dorsal and dorsolateral areas of the trunk were plucked, washed in saline buffer containing (in micromoles): NaCl 125.3, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D(+) glucose 5.6, and Tris-HCl buffer 5.0 (pH 7.3), individually im-

mersed in 96-well plates containing the same buffer and used for the next 4 h. Scales were initially treated with 10 μM melatonin for 5 min to induce melanophore aggregation (35) and then treated with agouti- or pcDNA3-conditioned medium (final dilution 1:20) for 10 min. Subsequently pcDNA3- or agouti-treated scales were incubated with 0.1 μM NDP-MSH (Bachem, Torrance, CA). After 10 min, NDP-MSH concentration was increased to 1.1 μM, and incubation continued for an additional 10 min. For quantification of pigment aggregation, pictures were taken at time 0 and 5 min after melatonin administration and 5 and 10 min after treatments with conditioned medium and melanocortin analog by using an DP50 camera (Olympus, Tokyo, Japan) attached to an Olympus IX71 inverted microscope. The perimeter of melatonin-responding melanophores was estimated using analySIS (Soft Imaging System GmbH, Germany).

Data analysis and statistics

Sequences were compiled in GenTool software package (BioTools Inc, Edmonton, CA) and compared with known agouti and AGRP sequences from the National Center for Biotechnology Information database by using BlastX. Sequence alignments were performed using Pileup from the GCG package and ClustalX 1.81 from Canadian Bioinformatics Resources (http://www.cbr.nrc.ca). The cleavage site for removal of the hydrophobic signal peptide was predicted using SignalP (version 1.1; http://www.cbs.dtu.dk/services/SignalP/). Binding and cAMP data were analyzed with Prism 3.0 software package (GraphPad). Differences in melanocyte perimeter were assayed by one-way ANOVA followed by Tukey's multiple range tests. Data are expressed as a percentage of the averaged perimeter at time 0. Statistical significance was considered at $P < 0.05$.

Results

Cloning and characterization of the goldfish ASP gene

By homology screening of a goldfish genomic DNA library with a mouse AGRP probe consisting of the exon 4 that encodes the C-terminal poly cysteine domain (mouse AGRP 86–131), two positive clones were isolated. The 5′ end fragment expanding 3.2 kb from clone 1.1.1.1 (European Molecular Biology Laboratory accession no. AJ845197) was sequenced and found to contain a fragment homologous to the fourth exon of mammalian ASP genes. Figure 1 shows the nucleotide sequence of the goldfish ASP gene. Analysis of the hydropathy profiles of the possible translations of the clone 1.1.1.1 enabled us to estimate the putative translation initiation site for ASP within the goldfish genomic sequence and assign primers for PCR. RT-PCR, using ventral skin cDNA as template, resulted in an expected size band of about 375 bp (Fig. 1). The so-called AGSP (EMBL accession no. AJ845198) clone displayed two substitutions *T*/*C* at positions 940 and 992 (Fig. 1). The former brings about a M/T amino acid substitution at position 19, inside the putative signal peptide of the goldfish ASP (Fig. 2). An additional band slightly longer than 500 bp was initially obtained by RT-PCR amplification of ventral skin using AgFwDTC/AgR2 primers. However, the same band was amplified when using cDNA from muscle, brain, and eye (Fig. 3). Two different-size clones, AgLF2 (EMBL accession no. AJ845200) and AgLF3 (EMBL accession no. AJ845201) of 532 and 506 bp long, respectively, were sequenced. Both clones were found to include the intron 2 and show *T*/*A* substitutions at positions 1132–1140 within the intron 2 (data not shown). Defective splicing of intron 2 generated a stop codon previous to the fourth exon translation. In addition, AgLF3 displayed a 24-bp deletion (2181–2204 bp) within the polycysteine C-terminal domain. No putative signal peptides in frame with exon 4 were detected, suggesting that AgFL2 and AgFL3 might be inactive alleles of the goldfish ASP gene.

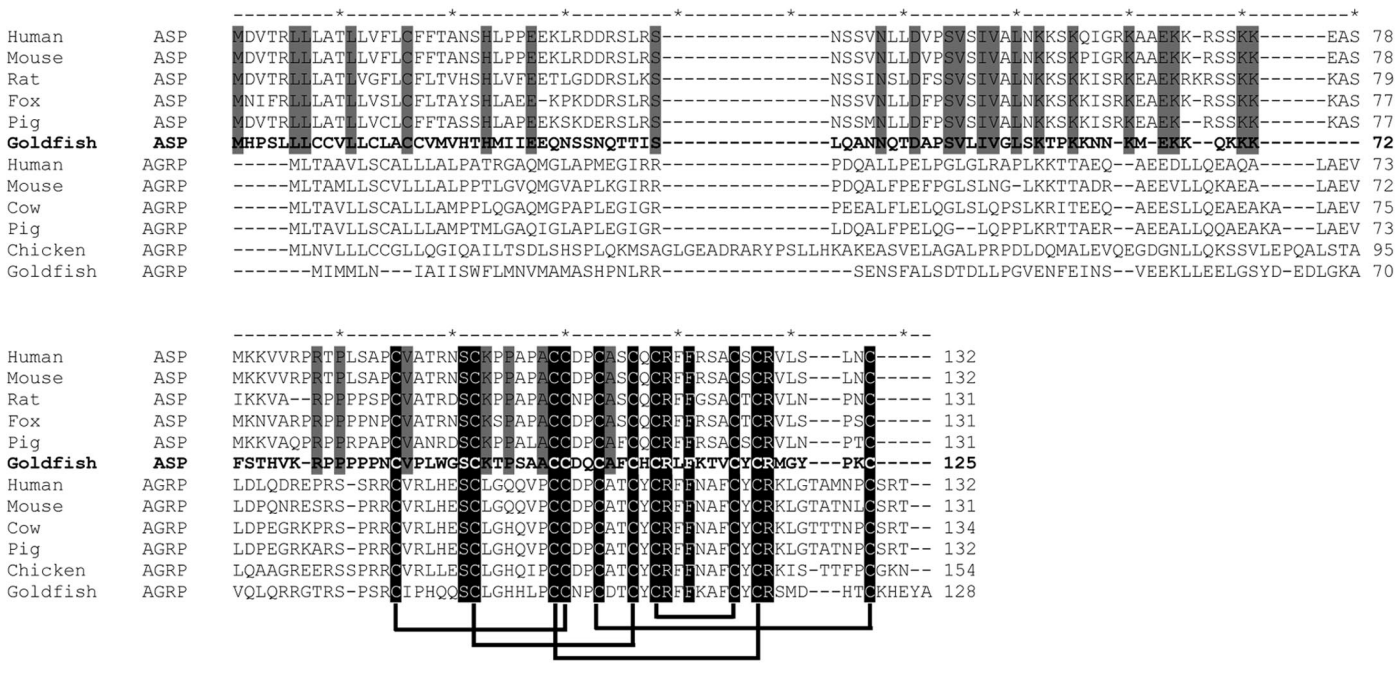

FIG. 2. Alignment of ASP [human (NM_001672), mouse (L06451), rat (NP_443211), pig (AJ251837), fox (Y09877), goldfish (AJ845197)] and AGRP [human (NM_001138), mouse (NM_007427), pig (AF177762), cow (NM_173983), chicken (AB029443), goldfish (AJ555493)] amino acid sequences. Goldfish ASP sequence is highlighted in *bold letters*. *Dashes* were introduced to improve alignment. *Black boxes* show amino acid residues conserved in all sequences. *Gray boxes* show residues only conserved in ASP sequences. *Lines* joining cysteine residues indicate putative disulfide bonds.

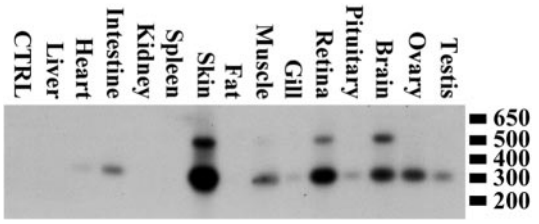

FIG. 3. Southern blot analysis of RT-PCRs showing tissue distribution of goldfish ASP mRNA expression. RT-PCR amplifications were carried out with primers set AgFwDTC and AgR2 (Fig. 1). The probe included the full coding region of the goldfish ASP. Control, No cDNA.

To obtain the complete cDNA sequence and determine the major transcription initiation site, RACE-PCR was performed in the 3′ and 5′ directions. A 981-bp band produced in 3′ RACE was cloned. Two different clones were characterized and found to display different sequences. The clone AG3L5 fully matched 3′ end of the genomic clone 1.1.1.1. The clone AG3L3 (EMBL accession no. AJ845202) exhibited two deletions within the proximal 3′ untranslated region (UTR) at positions 2290–2298 and 2359–2371 bp. In addition, clone AG3L3 displayed six substitutions within exon 4 at positions 2172 (*A*/*G*), 2197 (*T*/*C*), 2207 (*G*/*C*), 2255 (*T*/*C*), 2264 (*C*/*T*), and 2273 (*C*/*A*). Nucleotide substitutions at positions 2172, 2197, 2207, and 2273 bp bring about S/G, V/A, E/D, and N/K amino acid changes at positions 91, 99, 102, and 125, respectively (Fig. 2). The 5′ RACE resulted into three different size fragments (255, 286, and 379 bp) that were cloned into pGEM-T easy vector. The clone AG5–7 was 257 bp long, and the alignment to genomic sequence displayed an identical sequence within the alignment area. Similarly, the clone AG5–12 was 286 bp long, and the sequence was identical with AG5–7 but extended 31 bp upstream. The additional 31 bp fully matched to the sequence of the genomic clone. The clone AG5–2 was found to be identical with AG5–7, but it used a different splice donor site placed 124 bp downstream from which clones AG5–7 and AG5–12 splice intron 1.

To corroborate the alternative splicing detected as well as the sequences generated by 5′ and 3′ RACE, specific primers (AgFullFw and AgFullRev) targeting the respective UTRs were designed and used in RT-PCRs with ventral skin cDNA. RT-PCR resulted in two predicted size bands of 1174 and 1279 bp. The mRNAs sequences for goldfish ASP are estimated to be 1322 and 1445 bp and have an open reading frame encoding pre-ASP with 125 amino acids. The 5′ UTR are 126 and 249 bp for the short and long splice variants, respectively. The 3′ UTR is 821 bp long and contains five consensus polyadenylation signals AAUAAA. Comparison between cDNA and genomic sequences displayed four alignment blocks, denotating that goldfish ASP gene is organized into four exons separated by three introns. Goldfish ASP gene is a relatively small gene spanning 3097 bp from the start of the noncoding exon to the end of the 3′ UTR, including the last polyadenylation site. A TATA-box and SP1 binding sites (antisense orientation) were found at position −28 and −49, respectively. Exon 1 is alternatively spliced at positions 112 or 235 bp. This exon contains part of the 5′ UTR. Exon 2 (174 bp) includes the remaining part of the 5′ UTR, the region encoding the signal peptide and the first 31 amino acids of the putative mature peptide. Exon 2 ends with the nucleotide base G that constitutes the first base of a glycine codon after splicing. Exon 3 (53 bp) starts with the last two nucleotide bases GG of the latter glycine codon and encodes the following 27 amino acids of the mature peptide. Exon 4 (978 bp) encodes the remaining portion of the mature peptide, including the poly cysteine domain and contains the 3′ UTR. The introns are 635/758 (intron 1), 214 (intron 2), and 803 bp (intron 3) in length, and all three intron/exon junctions conform to the GT/AG rule of splicing.

The peptide precursor has the same organization as that of other species. The first 22 amino acids are estimated to constitute the signal peptide, which is followed by the 103 amino acids of the mature peptide. Three consecutive putative glycosylation sites were found within the highly basic N-terminal region. A proline-rich region and a poly cysteine C-terminal domain follow the basic N-terminal region. The polycysteine domain contains 10 cysteine residues with identical spatial pattern to that of agouti proteins, and, similar to mammalian ASP molecules, it does not exhibit a short amino acid extension after the 10th cysteine residue (Fig. 2). Goldfish ASP displays a low identity level to both tetrapod agouti and AGRP molecules, but the identity level is higher when compared with ASP than the AGRP proteins. Goldfish ASP is 35 and 17% identical with human agouti and AGRP, respectively. When considering equivalent taxons for agouti and AGRP sequences (human, mouse, and pig), goldfish ASP displays 45 and 22 constant positions, respectively.

Tissue distribution of the goldfish ASP mRNA

RT-PCRs with specific primers AgFwDTC and AgR2 resulted in a band of the expected size of about of 0.3 kb. The identity of the band was confirmed by Southern blot hybridization with a goldfish ASP probe including the full coding region (Fig. 3). Goldfish ASP mRNA was detected in the skin, brain, eye, and ovary and minor levels in testis, gill, muscle, intestine, heart, and pituitary. No hybridization signal was obtained in PCR using kidney, liver, spleen, and fat cDNA or water (control) as template. An unexpected bigger band was coamplified when using skin, brain, eye, and muscle cDNA as template. The homology to goldfish ASP was confirmed by Southern blot hybridization. The band was cloned, sequenced, and found to be an ASP form that does not splice intron 2. Sequence analysis suggests that it might be an inactive goldfish ASP allele (see previous section).

ASP mRNA expression in the goldfish skin

Two different bands about 1.5 and 2.2 kb were detected in ventral skin of five different red-pigmented fish, but no expression levels were observed in the dorsal skin of these same fish (Fig. 4). However, RT-PCR experiments detected very low expression levels in the dorsal skin (Fig. 5). The dorsal-ventral polarity in the expression of ASP mRNA was also detected in black dorsally pigmented and albino fish (data not shown). These two bands differ in size more than the goldfish ASP splicing forms, which are estimated to be about 1.3 and 1.4 kb. Considering the addition of the poly-A tail, these are reasonable sizes to fit to the smaller band observed in Northern blot. Given the tetraploid nature of goldfish (36),

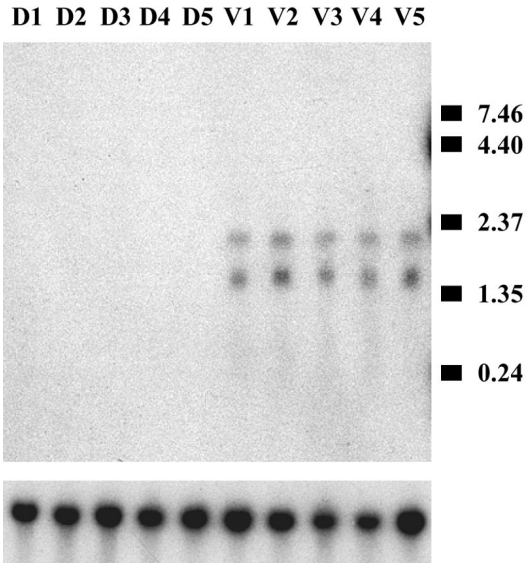

FIG. 4. Film autoradiogram of Northern-blotted total RNA from dorsal (D1-D5) and ventral skin (V1-V5) from five animals. A total 15 μg RNA were electrophoresed on a 1.5% agarose gel, containing 2.2 M formaldehyde, transferred onto a nylon membrane filter, and prehybridized during 3 h in 50% formamide, 5× SSPE, 1% SDS, and 5× Denhardt's solution, containing 20 μg/ml yeast tRNA type III at 42 C. The ^{32}P-labeled goldfish ASP probe was then added to fresh solution, and hybridization was carried out for 16 h under the same conditions. Final washes were performed in 0.1× SSPE/0.1% SDS at 65 C. The *numbers on the right* of the blot correspond to the localization of size marker RNAs (in kilobases). Two RNA bands of about 1.5 and 2.2 kb were detected in the ventral skin but never in the dorsal skin of the same animals.

the bigger size band might correspond to the second ASP copy of the goldfish genome.

Effects of goldfish ASP-conditioned medium on NDP-MSH binding and activation of MCRs

For pharmacological and functional characterization of the goldfish ASP, the coding region was ligated into an expression vector driven by cytomegalovirus promoter. The latter construct was transiently expressed in HEK-293 cells, and medium was collected at 24, 48, and 27 h post transfection. To corroborate goldfish ASP expression in the transient transfection experiments, Northern blot experiments using total RNA from HEK-293 cells at 24, 48, and 72 h post transfection were done. As shown in Fig. 6, very low ASP mRNA levels can be detected 24 h post transfection. Expression levels increased 48 and 72 h post transfection. According to these results, we subsequently used 48 h-conditioned medium for experiments involving binding and activation of the MCRs. Goldfish ASP-conditioned medium was tested in radioligand binding assays to intact cells expressing *Fugu* MC1R or goldfish MC4R. Conditioned medium from nontransfected or pcDNA-transfected cells were used as control. Results show that HEK-293 and pcDNA/HEK-293 conditioned medium had no effect in [^{125}I]NDP-MSH binding to both *Fugu* MC1R and goldfish MC4R. However, ASP-conditioned medium displaces [^{125}I]NDP-MSH binding at both tested receptors (Fig. 7, A and B). To test goldfish ASP effect on MCR activation as well as agonistic-antagonistic relationships, we studied intracellular accumulation of cAMP induced by binding of increasing concentrations of NDP-MSH either alone or in combination with ASP-conditioned medium. ASP-conditioned medium had no effect on intracellular cAMP accumulation at any dilution

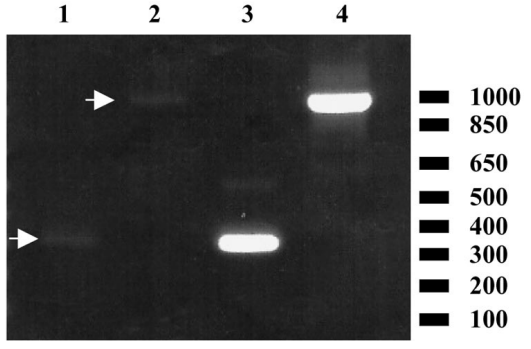

FIG. 5. Photography of an ethidium bromide-stained agarose gel showing RT-PCR products amplified from dorsal and ventral skin cDNAs of red-pigmented goldfish. Five hundred nanograms of mRNA were used in cDNA synthesis primed with dT-adapter (see *Materials and Methods* for sequence). Lanes 1 and 2, RT-PCR products from dorsal skin amplified with AgFWDTC-AgR1 or AgFWDTC-AgR2 primers, respectively. Lanes 3 and 4, RT-PCR products from ventral skin amplified with AgFWDTC-AgR1 or AgFWDTC-AgR2 primers, respectively. *Arrowheads* show amplification bands in cDNA form dorsal skin. See Fig. 1 for primer sequences.

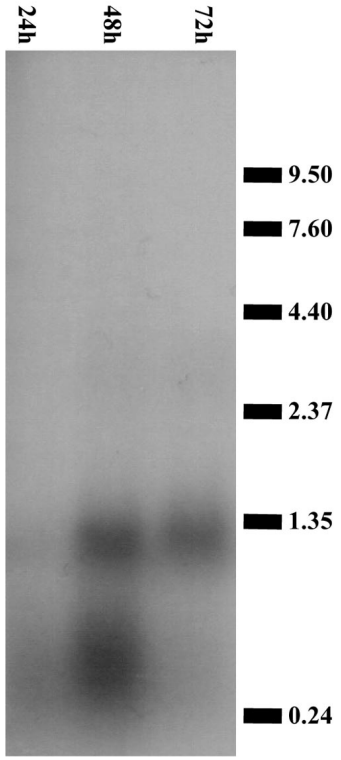

FIG. 6. Film autoradiogram of Northern-blotted total RNA from HEK-293 cells transiently transfected goldfish ASP cDNA into an eukaryotic expression vector driven by cytomegalovirus promoter (pcDNA3). Total RNA was extracted at 24, 48, and 72 h post transfection, and 10 μg RNA were electrophoresed and probed as before (see Fig. 4 for details). The *numbers on the right* of the blot correspond to the localization of size marker RNAs (in kilobases).

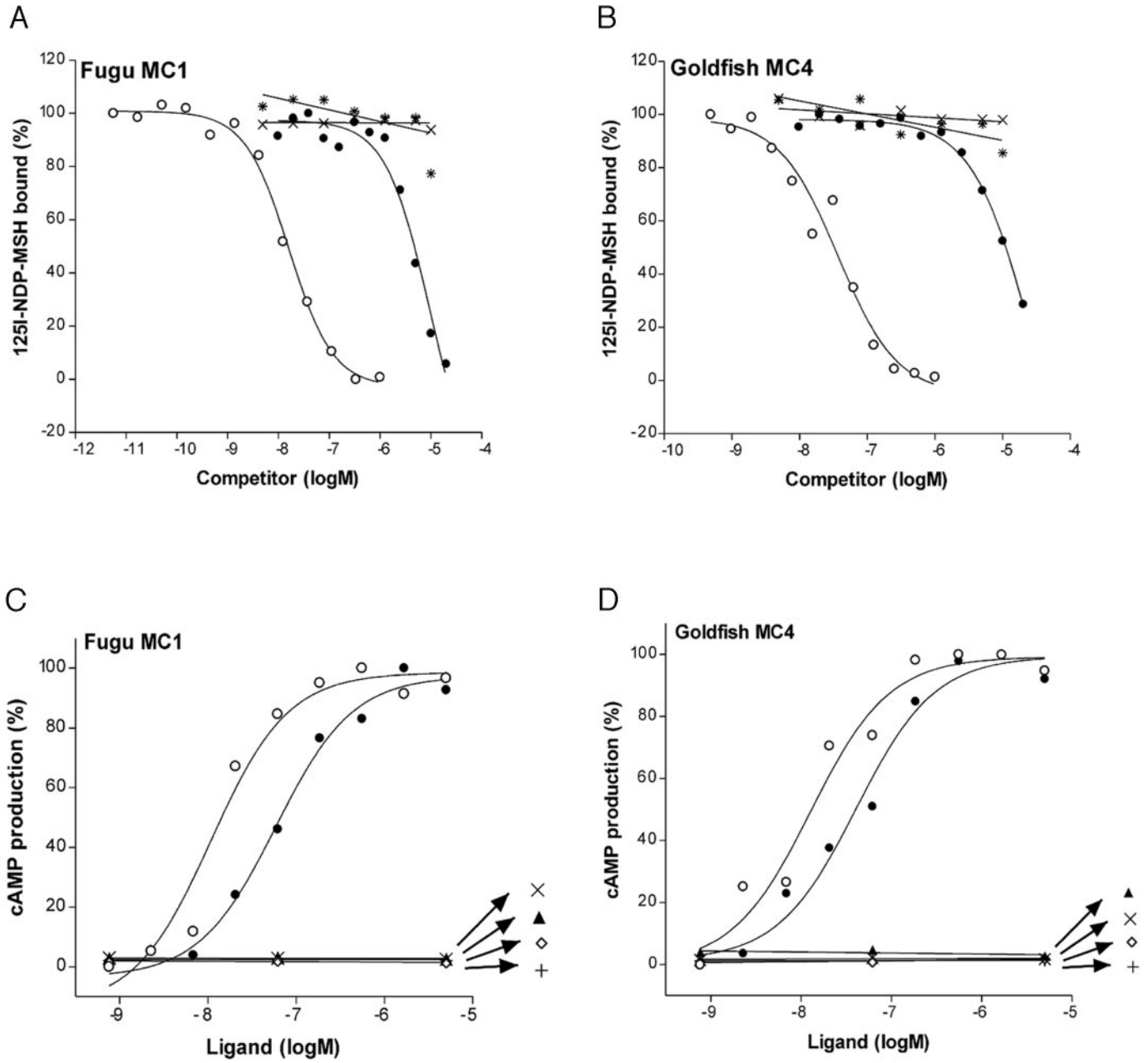

FIG. 7. A and B, Competition curves for *Fugu* MC1R (A) and goldfish MC4R (B) stably expressed in HEK-293 cells. The competition curves for NDP-MSH (○), goldfish ASP-conditioned medium (●) were obtained by using a fixed amount of 0.4 nM ^{125}I-NDP-MSH and serial dilutions of the nonlabeled competing peptide. ASP present in the medium harvested 48 h after transfection was used as competing ligand. Starting from the maximal amount of 50 μl, the medium was serially diluted 1:2 in 12 points. Conditioned medium from nontransfected HEK-293 (×) cells and HEK-293 transfected with intact pcDNA vector (*) diluted in the same way did not show any specific binding to *Fugu* MC1R and goldfish MC4R. C and D, Intracellular cAMP accumulation in HEK-293 cells stably expressing *Fugu* MC1R (C) and goldfish MC4R (D) in response to αMSH (○), αMSH plus constant amount of 50 μl ASP-conditioned medium (●), ASP-conditioned medium (▲), pcDNA-conditioned medium (◇) and HEK-293-conditioned medium (+). The basal level of cAMP is marked with ×.

tested on *Fugu* MC1R (Fig. 7C) and goldfish MC4R (Fig. 7D). In contrast, MSH-induced cAMP intracellular accumulation in cells expressing either *Fugu* MC1R (Fig. 7C) or goldfish MC4R (Fig. 7D) was attenuated when incubated with increasing concentrations of NDP-MSH and a fixed concentration of ASP-conditioned medium as would have been expected for a functional antagonist.

Effects of goldfish ASP-conditioned medium on medaka NDP-MSH-induced-melanophore dispersion

Melatonin induced a severe melanophore aggregation in scales from medaka adapted to black background within the first 5 min (Figs. 8, A and B, and 9, A and B). However, this effect was reversed by adding 0.1 μM NDP-MSH to the medium (Fig. 8C). Forty-eight hours pcDNA (Fig. 10) or agouti-

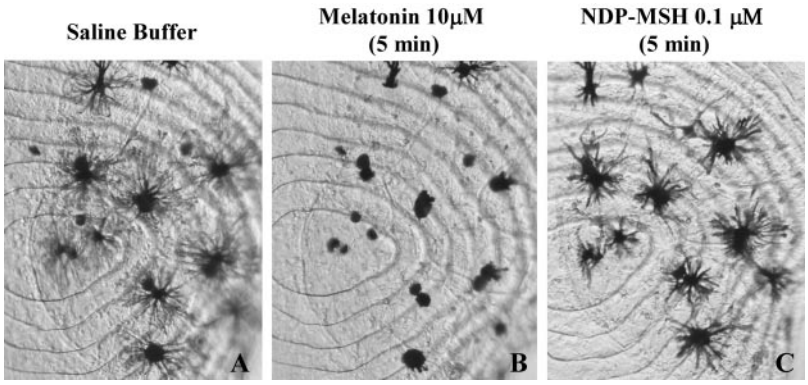

FIG. 8. Photomicrographs showing parts of same scale from medaka adapted to black background treated with saline buffer (A) showing melanophores (*black cells*) in state of dispersion, followed by 10 μM melatonin for 5 min (B) showing those in a state of aggregation and subsequently by 0.1 μM NDP-MSH (C) showing recovery of dispersion state after 5 min. Photomicrographs were taken with an inverted microscope (×80).

conditioned medium (Figs. 9C and 10) had no effect on melanophore aggregation in previously melatonin-treated scales. However, NDP-MSH (0.1 μM) induced a recovery in the melanophore perimeter at 5 min post treatment in scales previously treated with pcDNA-conditioned medium (Fig. 10). When scales were pretreated with ASP-conditioned medium, NDP-MSH (0.1 μM) was unable to induce pigment dispersion at 5 or 10 min after treatment (Figs. 9D and Fig. 10). An 11-fold increase in NDP-MSH concentration induced a partial recovery of melanophore perimeter extension in previously ASP-treated melanophores at 5 and 10 min after treatment (Figs. 9, E and F, and 10).

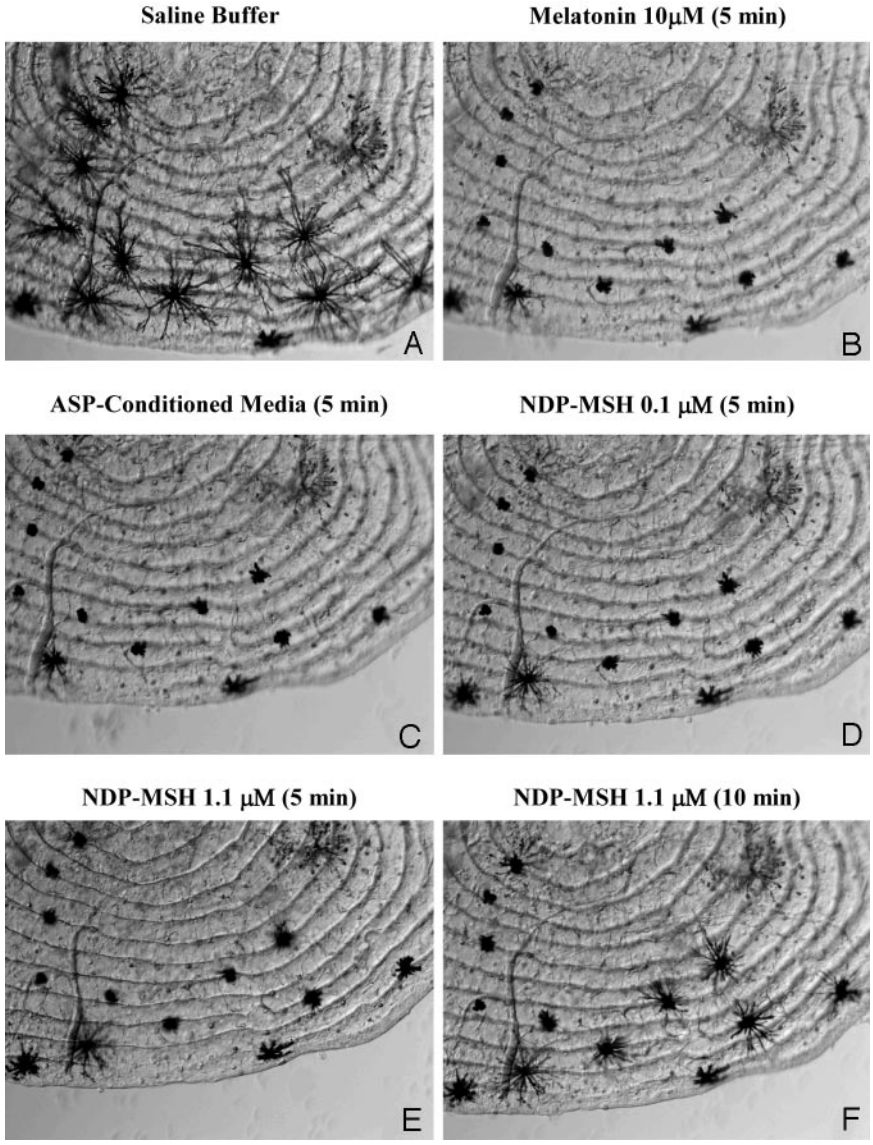

FIG. 9. Photomicrographs showing parts of same scale from medaka adapted to black background treated with saline buffer (A), followed by 10 μM melatonin for 5 min (B). subsequent addition of ASP-conditioned medium (1:20) for 5 min (C), 0.1 μM NDP-MSH for 5 min (D), and finally 1.1 μM NDP-MSH for 5 (E) and 10 min (F). *Black cells* are melanophores. Scales were photographed by using an Olympus DP50 camera attached to an Olympus IX71 inverted microscope (×40).

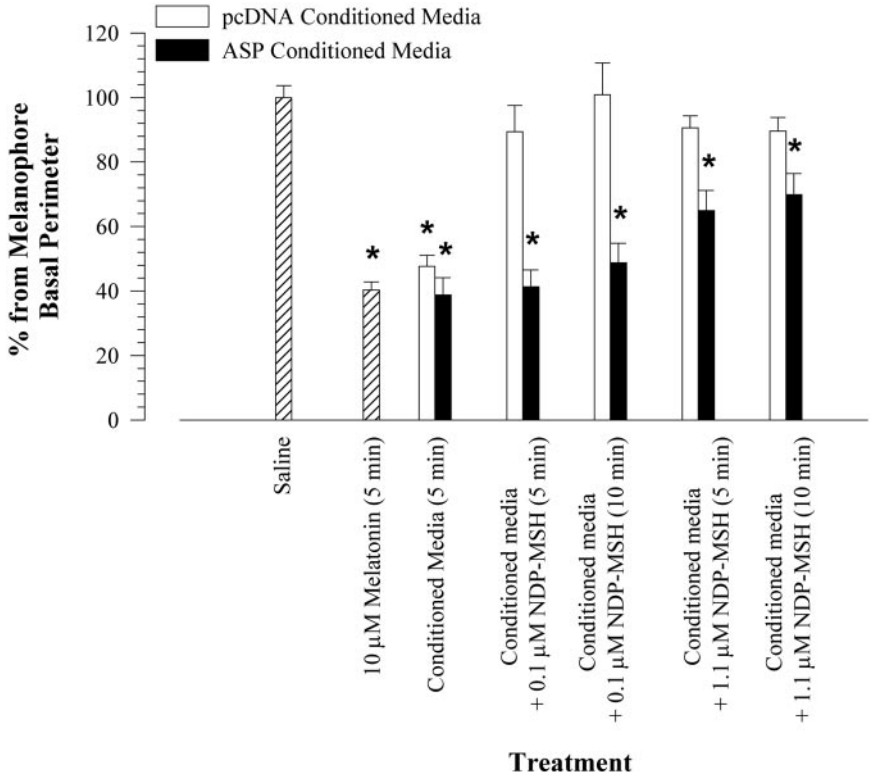

FIG. 10. Quantification of goldfish ASP effects on MSH-induced medaka melanophore dispersion. Scales were treated with saline buffer, followed by 10 μM melatonin for 5 min, subsequent addition of ASP-conditioned medium (1/20) for 5 min, 0.1 μM NDP-MSH for 5 and 10 min, and finally 1.1 μM NDP-MSH for 5 and 10 min. Each scale was photographed by using an Olympus DP50 camera attached to an Olympus IX71 inverted microscope, and the perimeter of each melatonin-responding melanophore was estimated by using analySIS (Soft Imaging System). Data ($18 \leq n \leq 90$) are expressed as a percentage of the basal perimeter (saline). *, Difference from saline levels ($P < 0.05$).

Discussion

In this paper, we demonstrated that the goldfish genome has a homologue gene to mammalian ASP, which has been described as a potent endogenous antagonist at MC1R and MC4R in mammals. These receptors have been shown to regulate melanogenesis (17) and energy homeostasis, respectively, in mouse (23). Goldfish ASP is mainly expressed in the ventral skin. Conditioned medium from goldfish ASP-expressing cells is able to displace [^{125}I]NDP-MSH binding at *Fugu* MC1R and goldfish MC4R, decrease NDP-MSH-induced cAMP intracellular accumulation at *Fugu* MC1R- and goldfish MC4R-expressing cell lines, and block NDP-MSH-induced melanin dispersion in medaka melanophore. All these data strongly support the ASP homology to the poikilotherm MIF as well as a role for ASP in the establishment of the dorsal-ventral pigment pattern in fish.

By homology screening of a goldfish genomic library, 5′, 3′ RACE and RT-PCR, the nucleotide and deduced amino acid sequence of goldfish ASP was determined. Both gene and precursor protein have a comparable organization to the mammalian counterpart. Goldfish ASP gene is organized into four exons separated by three small introns. Gene transcription generates two 1322- and 1445-bp-long mRNAs that encode an identical 125-amino acid precursor. These two different mRNAs result from the usage of an alternative splicing-donor site in the untranslated exon 1. In mouse, agouti gene has been reported to use four different exons, with exon 1 transcription being differentially regulated (14). The exons 1A and 1A′ are located approximately 120 kb upstream of the coding region. The alternative splicing of the exon 1A′ generates two different mRNA isoforms designated as 1A and 1A1A′. Therefore, the exon 1A′ does not seem to represent a transcription initiation site different from that of 1A. Both exons are specifically expressed in the ventral skin during the entire hair growth cycle of the light-bellied agouti (A^w/A^w) and black and tan (a^t/a^t) mice. The hair in the ventrum of the latter rodents contains only yellow pigment, whereas dorsal hairs display agouti or black color fur, respectively. The exons 1A and 1A′ are not expressed in the skin of the agouti mice (A/A) whose hairs contain a subapical band of yellow pigment on black background. Therefore, this transcription initiation site appears to govern the ventral-specific expression of agouti and by extension the dorsal-ventral pigmentary pattern. The exons 1B and 1C, which are separated by 40 bp and located approximately 18 kb upstream of the coding region, are expressed in both dorsal and ventral skin of the light-bellied agouti and agouti mice but only during the midportion of the hair growth cycle. The latter exons are not expressed in the a^t/a^t mouse whose dorsal fur is entirely black. The exons 1B and 1C appear to each represent independent sites of transcription initiation different from 1A and to be responsible for the yellow bands of the agouti fur (14).

We could not find additional exons, thus suggesting that goldfish ASP gene has only one transcription initiation site. However, more cloning experiments using different color strains and time-course developmental studies are required to discount the absence of alternative transcription initiation sites in the goldfish ASP gene. Goldfish short and long agouti isoforms may be homologous to the mice 1A and 1A1A′ isoforms. Both cases are not entirely equivalent because the mice 1A1A′ isoform is produced by an alternative splicing of

1A′, a 46-bp exon placed between exons 1A and 1B, whereas the long transcript of goldfish agouti is generated by the usage of a different splicing donor site in the exon 1. However, expression experiments by Northern blot demonstrated that goldfish ASP is basically expressed in the ventral skin, and only a very reduced expression level was detected in the dorsal skin by RT-PCR amplification. Because transcription initiation site associated to exon 1A in mice determines the dorsal-ventral pigmentary pattern, a morphotype conserved from goldfish to mouse, we suggest that the isolated goldfish ASP isoforms correspond to the mouse ASP 1A and/or ASP 1A1A′ forms.

By PCR amplification with primers targeting the coding region, we detected two mRNAs that do not process intron 2. This generates a premature stop codon that results in a truncated polypeptide completely lacking the translation of exons 3 and 4. One of these forms further exhibits a deletion within the C-terminal polycysteine domain encoding sequence. Because hydropathy profile analysis of the possible translations did not reveal any signal peptide in frame with the exon 4 translation, we suggest that the forms that do not process intron 2 are nonfunctional aberrant alleles of the goldfish ASP. However, further experiments are required to study their mRNA structure and phenotypic association. In mice, chemical- or radiation-induced mutations that delete the translation start site or produce aberrant agouti proteins are associated with nonagouti phenotype (37, 38). The allelic composition of the goldfish agouti locus was further supported by amplification of ventral skin cDNA with the primer set AgSPFw/AgR2. The so-called AGSP clone displayed two nucleotide substitutions when compared with the genomic DNA sequence that produced an M/T amino acid change at position 19 within the putative signal peptide. Mutations within the region encoding the signal peptide of mouse agouti are mainly associated with moderate hypomorphic color phenotypes (37, 38). In addition, we partially characterized a goldfish ASP mRNA that displayed two deletions within the proximal 3′ UTR and six changes within exon 4 that result in four amino acid substitutions. It is difficult to explain this high number of substitutions within exon 4 in terms of allelic composition. Because goldfish is a tetraploid species, it is possible that this mRNA constitutes a duplication of the ASP gene. Accordingly, Northern blot experiments detected two transcripts of about 1.5 and 2.2 kb in goldfish ventral skin.

The putative goldfish ASP precursor has structural characteristics of a secreted protein displaying a putative hydrophobic signal. Processing of the potential signal peptide produces a 103-amino acid mature protein that includes a N-terminal region, a basic central domain with a high proportion of lysine residues, a proline-rich region that immediately precedes the C-terminal poly cysteine domain. Whether additional posttranscriptional processing of goldfish ASP occurs is unknown, but Western analysis of the mouse agouti protein in several tissues suggests that mouse protein is not subjected to additional endoproteolytic processing (39). Three consecutive potential *N*-glycosylation sites were detected within the N-terminal region of the goldfish ASP. In mice glycosylation of ASP appears to be an important factor for the protein functionality, disruption of which partially reduces ASP activity in transgenic mice (40).

Similar to mammalian species, the basic domain of the goldfish ASP exhibits 10 lysine (K) residues but it only has one arginine (R) residue at position 79. This latter residue precedes the proline-rich domain, and it is fully conserved in all agouti sequences. The functionality of the N-terminal region and basic domain is reflected in the sequence conservation. Only eight amino acids are conserved in N-terminal region of the AGRP sequences, whereas 18 amino acids are fully conserved in mature ASP sequences. Six of these 18 amino acids are lysine residues placed within the basic domain. The biological role of the basic domain is poorly understood, but its integrity is basic for the full activity of the mouse ASP. Mutations deleting the entire 29-amino acid (41) or point mutations (38) within this domain of the mouse ASP significantly reduce the activity of the protein in transgenic animals. It has been suggested that the basic domain regulates ASP activity by influencing the biogenesis of the secreted protein or modulating the effective antagonism of MCRs (42, 43). The N-terminal region and/or this basic domain of mouse agouti has been shown to down-regulate MCR signaling in *Xenopus* melanophore (39) and is also thought to mediate low-affinity interactions with the product of the mahogany locus, a single transmembrane domain glycoprotein termed attractin that is expressed on the surface of the melanocyte and functions as a low-affinity receptor of agouti protein (44).

Whereas mutations within the N-terminal region including the basic domain tend to produce color hypomorphic phenotypes in transgenic mice, alterations within the polycysteine C-terminal domain generally induce loss of function phenotypes (38, 40). This suggests that the polycysteine C-terminal domain is the most important region from the functional point of view. Accordingly, it has been demonstrated that the cysteine rich C-terminal domain of agouti protein is sufficient for αMSH competitive antagonism on *Xenopus* melanophores (39) and αMSH binding inhibition on human MC1R and MC4R (42, 45, 46). The polycysteine domain is the most conserved region between AGRPs and ASPs, including goldfish orthologs. Ten cysteine residues compose this domain. Spacing of the 10 cysteines is absolutely conserved in ASP orthologs, and the goldfish sequence is not an exception. Nine of the 10 cysteines are also spatially conserved in the C-terminal domain of AGRP proteins. This cysteine pattern resembles that of the conotoxins and agatoxins, suggesting that agouti and AGRP adopt an inhibitory cysteine knot fold.

Structural studies have demonstrated that five disulfide bridges between cysteine residues, C87-C102, C94-C108, C101–119, C105–129, and C110-C117, stabilize the human AGRP molecule (47–50). The structural integrity of the cysteine knot is basic for the agouti activity. Transgenic mouse expressing mutant agouti cDNAs in which each of all 10 cysteine residues of has been substituted with serine displays black fur and normal body weight. Individual substitutions showed that eight of the 10 cysteine residues are functionally essential, and those mutations in the pair of cysteine residues that anchor the final seven residues result in only partial loss

of the *in vivo* activity (40). This RFF triplet is fully conserved in all AGRP and mammalian ASP sequences, but surprisingly the first phenylalanine (F) is replaced by a leucine (L) residue in goldfish ASP, thus resulting in an RLF triplet. The same amino acid replacement was found in the putative second copy of goldfish ASP and a partially characterized AGRP/ASP-like molecule reported in *Fugu* (28). This small motif shows chemical properties resembling the core sequence HFRW in MSHs (51) and has been proposed to form a primary contact point for the ligand-receptor interaction (47, 51). In the human AGRP, the substitution of the first phenylalanine (F112) in the RFF triplet by another hydrophobic amino acid alanine (F112A) results in a 50-fold reduction in the affinity for human MC3R and MC4R (45). Additional studies are needed to evaluate the antagonism of goldfish ASP to mammalian receptors.

Radioligand experiments demonstrated that ASP-conditioned medium displaces binding of NDP-MSH to *Fugu* MC1R and goldfish MC4R. However, pcDNA3-conditioned medium was unable to displace melanocortin agonist binding to any tested receptor. All tested melanocortin receptors were positively coupled to the cAMP-signaling pathway, in response to the nonselective melanocortin agonist NDP-MSH. In cells that express *Fugu* MC1R and goldfish MC4R, ASP-conditioned medium induced a parallel rightward shift in the dose response curves for NDP-MSH-stimulated cAMP intracellular accumulation. The latter effect was not observed when using pcDNA3-conditioned medium. This parallel rightward shift is consistent with a reduction in the NDP-MSH-induced cAMP production and by extension with an endogenous antagonism. This demonstrates that ASP is a functional and suitable antagonist at *Fugu* MC1R and goldfish MC4R. Mammalian ASP has been reported to antagonize αMSH effects at mouse (22) and human (46) MC1R and MC4R. Mouse ASP had relatively weak effects at human (46) MC3R and MC5R but no effects at rat MC3R and mouse MC5R (22).

Goldfish ASP antagonism to melanocortin receptors was further supported by the inhibitory effects of ASP-conditioned medium on NDP-MSH-induced melanin dispersion in medaka scale melanophores. Melanocortin receptor subtypes expressed in fish melanophores are unknown. We previously described weak MC4R and MC5R expression in goldfish skin (33, 52), but on this basis it is not possible to predict that MC1R is the main melanocortin receptor in fish chromatophores. The effects of ASP on melanocortin-induced melanin dispersion further support a role for ASP on fish chromatophore physiology and by extension in the fish color pattern.

Central ASP antagonist at MC4R is responsible for the metabolic disorders observed in agouti yellow mice (A^y). This metabolic syndrome is characterized by hyperphagia and maturity-onset obesity (51). Ectopic ASP expression in the mouse brain emulates central AGRP expression, thus antagonizing the inhibitory tone on food intake imposed by melanocortins through MC4R signaling (24, 25, 51). We previously reported that central MC4R is widely expressed in the forebrain and is involved in the control of energy balance in the goldfish (33). Furthermore, fasting severely upregulates hypothalamic AGRP expression (26), an endogenous goldfish MC4R antagonist (Cerdá-Reverter, J. M., T. Haitina, and H. B. Schiöth, unpublished results). The exclusion of ASP expression from the mammalian central nervous system does not seem to apply in goldfish because RT-PCR/Southern blot experiments detected ASP expression in brain, particularly in the telencephalon/preoptic area (data not shown). Therefore, the regulation of central melanocortin signaling cannot be exclusively attributed to the tandem of proopiomelanocortin/AGRP in the goldfish brain.

Peripheral ASP antagonism at MC1R regulates the eumelanin to pheomelanin synthesis ratio at the hair-follicle melanophore, and it is thus responsible for the dorsal-ventral pigment pattern in mouse (14). In addition, ASP inhibits the differentiation and proliferation of melanoblast by decreasing αMSH-induced expression of microphthalmia (MIFT), a transcription factor belonging to the basic helix-loop-helix family as well as the inhibiting MIFT binding to the M box in melanogenic gene promoters (18, 19). This transcription factor has been suggested to mediate the effects of cAMP on tyrosinase, the rate-limiting enzyme of the melanogenesis, tyrosinase-related protein 1 and 2 promoter activities through binding to M box. Properties of mammalian ASP hardly resemble those of poikilotherm MIF. The latter peptide is also a local diffusible factor mainly produced by cells in the amphibian and fish ventral skin (13). Amphibian MIF interacts with melanocortin system by inhibiting αMSH-stimulated melanization in neural tube explants from *Xenopus* (11) and decreasing αMSH-stimulated activity of melanogenic enzymes as tyrosinase and dopachrome tautomerase in murine melanoma cell lines (12).

Our results demonstrate that goldfish ASP resembles poikilotherm MIF because it is mainly expressed in the ventral skin; interacts with the melanocortin system as an endogenous antagonist, thus decreasing NDP-MSH-induced intracellular accumulation of cAMP at *Fugu* MC1R and goldfish MC4R expressing cell lines; and blocks NDP-MSH effects in medaka melanophore. Goldfish ASP expression was detected in the ventral skin of the all three color phenotypes studied, *i.e.* orange and black dorsally pigmented and albino fish, but not in the dorsal skin. These data strongly suggest that ASP is mainly involved in the establishment of the dorsal-ventral pigment pattern in fish by directing chromatoblast differentiation to iridophores and inhibiting differentiation to melanophores and/or any other chromatophore subtypes. Accordingly it has been demonstrated that conditioned medium from catfish ventral skin and semipurified amphibian MIF inhibits melanoblast differentiation but stimulates or supports iridophore proliferation (7, 9, 10). Supporting these data, it has been recently reported that microphthalmia-related transcription factor is sufficient to direct differentiation of medaka embryonic stem-like cells to melanocytes (53). It is therefore plausible that ASP inhibits MIF-related transcription factor because it makes in mammalian systems deviating chromatoblast differentiation to iridophore. However, additional experiments are required to study ASP effects on MIFT-related transcription factor expression, chromatoblast differentiation, and melanogenic enzyme activities in fish.

Acknowledgments

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Address all correspondence and requests for reprints to: Dr. J. M. Cerdá-Reverter, Department of Fish Reproductive Physiology, Instituto de Acuicultura de Torre de la Sal, 12595 Torre de la Sal, Ribera de Cabanes, Castellón, Spain. E-mail cerdarev@iats.csic.es.

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