Recombinant novel pituitary adenylate cyclase-activating polypeptide from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth-promoting factor in low vertebrates

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Abstract

Nowadays, the studies of pituitary adenylate cyclase-activating polypeptide (PACAP)-related peptide (PRP) and PACAP in non-mammalian vertebrates, especially in fish, have paid attention mainly to the localization, cloning, and structural evolution of the peptides, but very little is known about its biological functions as growth-promoting factors in low vertebrates. In this work, we have cloned and characterized the PRP/PACAP cDNA from the commercially important North African catfish *Clarias gariepinus*. The sequence obtained agrees with the higher conservation of PACAP than of PRP peptide sequences. We have reported for the first

time the recombinant expression of fish PRP and PACAP in mammalian cells and bacteria and also demonstrated that the growth rate of fish is enhanced by both PRP and PACAP recombinant peptides. The results obtained *in vivo* in three different fish species, catfish (*C. gariepinus*), tilapia (*Oreochromis niloticus*), and carp (*Cyprinus carpio*) support the finding that PACAP rather than PRP plays a primordial role in growth control in teleost fish. This finding could help to elucidate the neuroendocrine axis proposed to explain the hypothalamic regulation of growth in non-mammalian vertebrates.

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first identified in the ovine hypothalamus due to its ability to stimulate cAMP formation in rat anterior pituitary cells (Miyata *et al.* 1989). This peptide belongs to the secretin/glucagon superfamily of regulatory neuropeptides that also includes secretin, glucagon, glucagon-like peptides 1 and 2, vasoactive intestinal peptide, histidine–methionine peptide, histidine–isoleucine peptide, and glucose-dependent insulinotropic peptide (Campbell & Scanes 1992).

In mammals, PACAP and growth hormone-releasing hormone (GHRH) are encoded by separate genes on separate chromosomes (Hosoya *et al.* 1992, Perez Jurado *et al.* 1994). In non-mammalian vertebrates (Parker *et al.* 1993, McRory *et al.* 1995, 1997, Alexandre *et al.* 2000) and protochordates (McRory & Sherwood 1997), PACAP and GHRH-like peptide were believed to be encoded by the same gene and hence processed from the same transcript and prepropolypeptide. However, a gene encoding only GHRH in non-mammalian vertebrates was recently discovered in goldfish, zebrafish, and African clawed frog (Lee *et al.* 2007), and a shortened precursor cDNA encoding only for PACAP has been isolated in catfish (McRory *et al.* 1995), chicken

(McRory *et al.* 1997), and frog (Alexandre *et al.* 2000). Recent reports of an authentic GHRH and its receptor gene in frog and fish, based on sequence comparisons, phylogenetic studies and chromosomal localizations in vertebrates has demonstrated that the previously named GHRH-like peptides are homologs of mammalian PACAP-related peptides (PRPs). Taking this information into account, Lee *et al.* (2007) proposed that henceforth GHRH-like peptides be renamed as PRP.

Two molecular forms of PACAP with 38 and 27 amino acids respectively were originally isolated from sheep hypothalamus (Miyata *et al.* 1989, 1990). The sequence of PACAP has been remarkably well preserved from tunicate to human. In particular, the sequence of PACAP38 is identical in all mammalian species studied so far (Gonzalez *et al.* 1998). By contrast, PRP is moderately conserved. The high conservation of the PACAP sequence indicates that PACAP fulfils important biological functions from fish to mammals.

Studies examining the effect of PACAP on GH secretion have shown contradictory results (Montero *et al.* 2000). Some studies suggest that PACAP stimulates GH release *in vitro* in pituitary cells from rat (Hart *et al.* 1992, Jarry *et al.* 1992), sheep (Sawangjaroen *et al.* 1997), bovine (Hashizume *et al.* 1994), swine (Martinez-Fuentes *et al.* 1998), and fish (Parker *et al.* 1997, Montero *et al.* 1998, Wong *et al.* 1998, 2005,

Xiao *et al.* 2002, Sze *et al.* 2007). By contrast, other studies have reported that PACAP has no effect on GH secretion (Jarry *et al.* 1992, Sawangjaroen & Curlewis 1994). *In vivo* studies have demonstrated that PACAP increases plasma GH levels in rat (Jarry *et al.* 1992), but not in humans (Chiodera *et al.* 1996), suggesting that the GH-releasing effect of PACAP in mammals may be species-specific. The stimulatory effect of GHRH on GH secretion in mammals has been well identified (Guillemin *et al.* 1982, Bertherat *et al.* 1995); however, the presence of genuine GHRH peptides in fish was only recently demonstrated (Lee *et al.* 2007). This novel fish GHRH was able to stimulate GH secretion in fish pituitary cells *in vitro* (Lee *et al.* 2007). Additional studies are necessary to fully characterize the role of this novel neuropeptide in the GH regulation. On the other hand, the peptide recently renamed as a PRP has been reported to exert a weak-to-modest stimulatory effect on GH secretion from fish pituitary *in vitro* (Luo *et al.* 1990, Vaughan *et al.* 1992, Parker *et al.* 1997, Lee *et al.* 2007). In the glucagon superfamily, PRP is the only peptide whose function has remained undefined since its discovery more than a decade ago (Tam *et al.* 2007). Despite repeated efforts, a PRP receptor has been isolated only in goldfish (Chan *et al.* 1998). It is highly possible that PRP plays a physiological role in non-mammalian vertebrates, whereas its function has been lost in mammals due to the loss of its receptor in the genome (Lee *et al.* 2007).

To date, the studies of PRP and PACAP in non-mammalian vertebrates, especially in fish, have focused primarily on the localization, cloning, and structural evolution of these peptides and very little is yet known about their biological functions as growth factors *in vivo*.

In this work, we have cloned and characterized the PRP/PACAP cDNA from the commercially important African catfish (*Clarias gariepinus*). We have expressed both peptides in mammalian cells and bacteria, and demonstrated for the first time that both PACAP and PRP play a physiological role in growth control in teleost fish.

We have focused our study specifically to examine the possible use of recombinant PACAP as a new growth-promoting supplement in fish. Such a finding could be used as a powerful biotechnology tool to improve the fish growth in aquaculture.

Materials and Methods

Animals

African catfish (*C. gariepinus*) and tilapia (*Oreochromis niloticus*) were provided by the Mamposton Aquaculture Research Station, Havana, Cuba, and were kept alive in aerated freshwater under natural photoperiod. Water temperature was maintained between 28 °C and 30 °C. Carp (*Cyprinus carpio*) were provided by Nacari Company, Havana, Cuba, and were kept as described above. All animal experiments were previously approved by the Ethics Committee of the Center for Genetic Engineering and Biotechnology, Havana, Cuba.

Cell cultures

Baby hamster kidney-21 cells (BHK; ATCC CRL-8544) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) and were maintained in an agitated cultivation system at 5% CO₂ atmosphere with a relative humidity of 95%. The cells were cultivated every 3–4 days at a seeding density of 2.5×10^5 cells/ml.

Primer designs

The specific primers used to amplify the *C. gariepinus* cDNA corresponding to PRP/PACAP (PAC) were F-PRP/PAC and R-PRP/PAC based on *Ictalurus punctatus* PRP/PACAP cDNA (GenBank accession number AF321243). Primers for signal peptide-PRP (SP-PRP) to be cloned into mammalian expression vector pTargetT (Promega) were F-PRP/PAC and R-CMV-PRP; for PACAP without signal peptide to be cloned into pTargetT vector were F-CMV-PACAP and R-CMV-PACAP; for SP-PRP to be cloned immediately adjacent to the 5'-PACAP region were F-SP and R-SP; and for PACAP to be cloned into *Escherichia coli* expression vector pTYB1 (New England Biolabs, USA) were F-pTYB-PRP, R-pTYB-PRP, F-CMV-PACAP, and R-pTYB-PACAP. The specific primers (F- β -actin and R- β -actin), based on tilapia (*Oreochromis mossambicus*) β -actin sequence (GenBank accession number AB037865), were used to verify the quality of synthesized cDNAs. The primer sequences with the restriction endonuclease sites included for the cloning are given in Table 1.

RNA isolation

Total RNA from *C. gariepinus* brain was extracted using RNeasy total RNA isolation system (Promega) and was quantified by measuring the absorbance at 260 nm and stored at –80 °C until use.

Isolation of cDNA encoding *C. gariepinus* PRP/PACAP precursor

Five micrograms of total RNA from *C. gariepinus* brain and 1 μ l oligo (dT)₁₅ (0.5 μ g/ μ l) were incubated at 70 °C for 5 min and placed on ice. The reaction was carried out in a total volume of 20 μ l with 1 $\times$ avian myeloblastosis virus (AMV)-reverse transcriptase (RT) buffer, 1 μ M of each dNTP, 1 u/ μ l RNase inhibitor, and 15 u/ μ g AMV-RT (Promega); incubated at 42 °C for 30 min and at 95 °C for 5 min; and then diluted to a final volume of 100 μ l in nuclease-free water. The quality of synthesized cDNA was evaluated by PCR amplification of *C. gariepinus* β -actin partial cDNA using the specific primers F- β -actin and R- β -actin (Table 1). The PCRs were carried out using 20 μ l five times diluted RT mixture, the appropriate PCR buffer volume at a final concentration of 1 $\times$ (100 mM Tris-HCl, 500 mM KCl (pH 8.3), 1.5 mM MgCl₂), 50 pmol of each primer based on the *I. punctatus* PRP/PACAP cDNA

Table 1 Primer sequences

Primer name	Primer sequence ^a	Restriction endonuclease enzyme
F-PRP/PAC	5'-gcag ccatgg ccaatctagtagactac-3'	NcoI
R-PRP/PAC	5'- ggaattc ctttaatggcttgactctgacat-3'	EcoRI
R-CMV-PRP	5'-cg ggatc cttacgacagtggtccgagtcctcc-3' ^b	BamHI
F-CMV-PAC	5'-catc catatg cactcggacggcattttcacgg-3'	NdeI
R-CMV-PAC	5'-cg ggatc cttattgtttctaaacctctgtctgtacct-3'	BamHI
F-SP	5'-ctag ctagc atggccaatctagtagactac-3'	NheI
R-SP	5'-gtttcggtcttctctcggg-3'	— ^c
F-pTYB-PRP	5'-catc catatg catgcagacggattgttagatagag-3'	NdeI
R-pTYB-PRP	5'- tgctcttccgc acagactggctccgagtcctct-3'	SapI
R-pTYB-PAC	5'- tgctcttccgc attgtttctaaacctctgtctgtacct-3'	SapI
F-β-actin	5'-acactgtgcccatctacgaggg-3'	— ^c
R-β-actin	5'-cgatccagacggagtatttacgc-3'	— ^c

^aRestriction endonuclease sites are in bold.
^bThe triplet codon is indicated with a single underline.
^cPrimers without restriction endonuclease site.

sequence, and 2.5 units of *Taq* DNA polymerase (Heber Biotec SA, Havana, Cuba). The amplification of *C. gariepinus* PRP/PACAP cDNA was carried out for 30 cycles as follows: denaturation for 30 s at 95 °C, 1 min annealing at 68 °C, and 1 min extension at 72 °C, followed by an initial denaturation at 95 °C for 5 min. Amplification was completed with an additional extension step at 72 °C for 5 min. The PCR products were analyzed in 1.5% agarose gel electrophoresis.

Cloning of the PRP and PACAP cDNA into mammalian expression vectors

The sequence containing both the signal peptide and mature PRP was isolated by PCR using the *C. gariepinus* PRP/PACAP cDNA precursor as template. The cDNA encoding only for the mature PACAP was also amplified as described above. The signal peptide sequence from *C. gariepinus* PRP/PACAP precursor was amplified and cloned into pTargetT expression vector (Promega) immediately adjacent to the 5'-PACAP region. The specific primers F-PRP/PAC, R-CMV-PRP, F-CMV-PACAP, and R-CMV-PACAP were used in order to amplify the SP-PRP and PACAP cDNA sequences respectively, and to include the restriction endonuclease sites necessary for cloning into the eukaryotic expression vector pTargetT (Table 1). The signal peptide amplification for SP-PACAP vector construction was done using the F-SP and R-SP primers (Table 1). The final vectors (pCMV-SP-PRP and pCMV-SP-PACAP) were tested for both restriction endonuclease site analysis and DNA sequencing.

Sequence analysis

The plasmid DNAs were purified using a Mini Preps kit (Promega), and the inserts were completely sequenced by MacroGen (Seoul, South Korea) using the specific primers annealing to the regions of the vector flanking the multiple cloning sites.

Sequence analysis was carried out using BLASTX (<http://www.ncbi.nlm.nih.gov/blast/blastx>) or CLUSTALW (<http://www.ebi.ac.uk/Tools/clustalw>).

Transfection of BHK-21 cells

BHK-21 cells were transfected with the pCMV-SP-PRP and pCMV-SP-PACAP vectors, previously linearized with BamHI restriction enzyme, using a stock solution of linear 25 kDa polyethylenimine (PEI; Aldrich). The PEI transfection agent was prepared in water at a final concentration of 1 mg/ml and the pH adjusted to 6.8 with HCl. This solution was sterilized using a 0.22 µm filter and stored at -80 °C until use.

Typically, 1.5 × 10⁶ cells in DMEM were plated onto a 100 mm dish, 1 day prior to the transfection. For transfecting, the cells were washed once with DMEM, suspended in the same medium at 1 × 10⁶ to 2 × 10⁶ cells/ml and then dispensed into 12-well plates (1 ml). Meanwhile, 7 µg of each vector DNA and 500 ng pCMV-GFP (the green fluorescent protein DNA sequence cloned previously into pTargetT vector) in 150 mM NaCl were mixed with PEI at a 1:1 ratio and incubated at room temperature for 10 min. The DNA-PEI mix was added to each well and the plates incubated for 4 h at 37 °C, 5% CO₂ atmosphere, and 95% humidity. Following this the cells were diluted with 1 ml DMEM and incubated for 48 h. The transfected cells were harvested by trypsinization with a solution containing 0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution (Gibco, Invitrogen). The trypsinization was stopped by adding DMEM supplemented with 10% inactivated horse serum (Sigma). The harvested cells were washed three times with 1 ml Hanks' balanced salt solution and then frozen in liquid nitrogen until use.

The co-transfection of the pCMV-GFP plasmid with each of the DNA vectors, as described above, was used to evaluate the transfection efficiency. Untransfected as well as pTargetT

vector-transfected plates (mock-transfected cells) were used as negative controls.

Static hemipituitary gland culture

A sample of juvenile tilapias (*O. niloticus*) was randomly taken from the tank in the morning prior to feeding (0830–1030 h). After anesthetizing with the methanesulfonate salt of 3-aminobenzoic acid ethyl ester (Sigma) dissolved in water, pituitary glands were immediately removed and placed in an ice-cold saline solution (4.17 mM NaHCO₃, 0.1% BSA (pH 7.5), diluted to 80%). Afterward, individual pituitary glands were dissected along the sagittal axis into two equal parts. The hemipituitaries were then rinsed three times in the 80% saline solution described above, placed individually into single wells of 24-well tissue culture plates (Corning Costar, Corning, NY, USA), and cultured in 1 ml 80% saline solution for 1 h at 30 °C on a shaker platform. The medium was then replaced with 1 ml M199 (Gibco; supplemented with 4.17 mM NaHCO₃, 25 mM HEPES, 0.1% BSA, 0.7 mM L-glutamine, 100 µg/ml streptomycin, 100 u/ml penicillin-G (pH 7.5), and 5% inactivated horse serum), and incubated for 4 h at 30 °C to establish basal GH release. Prior to the assays, the medium was replaced with either supplemented M199 (control wells) or supplemented M199 containing the test substance (treatment wells), but both without serum. Treatments consisted of adding equal volume (300 µl) of the culture supernatants of the pCMV-SP-PRP-, pCMV-SP-PACAP-, and pTarget-transfected BHK-21 cells. The synthetic GH secretagogue (GHRP-6; Lipotec, Barcelona, Spain) was also included as positive control. The medium was removed after 24 and 48 h of incubation and stored at –20 °C prior to GH assays. Treatments were tested in duplicate, using three to four fish pituitaries for each test. This experiment was repeated at least two times.

GH assays

The GH secreted *in vitro* was measured using a non-competitive ELISA. The 96-well MaxiSorp plates (Nalge Nunc International, Roskilde, Denmark) were coated 3 h at 37 °C with anti-tilapia GH monoclonal antibody 1 (tiGH1 mAb; CIGB, Cuba) at 10 µg/ml in 0.05 M carbonate buffer (pH 9.6, 100 µl/well). The plates were washed two times with PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, and 0.05% Tween 20 (pH 7.3)) and blocked with 3% skim milk (Oxoid, Cambridge, UK) in PBS 1× (200 µl/well) by 1 h at 37 °C. A standard tilapia GH curve in the range of 32–0.5 ng/ml was obtained by twofold dilutions in 0.5% skim milk in PBS 1× and it was dispensed by duplicate in the same plate. Test samples were diluted at a ratio of 1:2 as described above and were incubated on the plates overnight at 4 °C. After washing the plates for four times with PBS-T, 100 µl horseradish peroxidase (HRP)-tiGH2 mAb conjugate (CIGB), diluted at a ratio of 1:15 000 in PBS 1×, containing 0.5% skim milk were added to each well. The

plates were incubated for 1 h at 37 °C and then washed eight times with PBS-T. Then the substrate buffer (0.2 M Na₂HPO₄, 0.1 M citric acid (pH 5.0)) containing 0.5 mg/ml *ortho*-phenylenediamine and 5 µl 30% H₂O₂ were added (100 µl/well). The reaction was stopped 15 min later by adding 50 µl per well 2.5 M sulfuric acid. The absorbance was measured at 492 nm using the Titertek Multiskan Plus spectrophotometer.

Cloning of the PRP and PACAP cDNA into *E. coli* expression vectors

The mature PRP and PACAP cDNA sequences amplified by PCR using the PRP/PACAP cDNA precursor as template and the specific primers F-pTYB-PRP, R-pTYB-PRP, F-CMV-PACAP, and R-pTYB-PACAP respectively (Table 1), were subcloned into pGEM-T Easy vector using the pGEM-T Easy vector system I kit (Promega). After double digestion with NdeI and SapI, the purified DNA fragments were directly ligated to NdeI/SapI double digested pTYB1 expression vector. The pTYB vectors are commonly used for cloning and the expression of recombinant proteins in *E. coli*. The pTYB1 is a C-terminal fusion vector in which the C-terminus of the target protein is fused to the intein tag. This vector is part of the Intein-Mediated Purification with an Affinity Chitin-Binding Tag (IMPACT) system (New England Biolabs, Beverly, MA, USA). The insertion of the PRP and PACAP genes into the pTYB1 vector (pTYB1-PRP and pTYB1-PACAP) was verified by both restriction endonuclease sites analysis and DNA sequencing.

Expression of the recombinant mature PRP and PACAP in bacteria

The pTYB1 vector uses a T7 promoter-driven system to achieve high levels of expression and tight transcriptional control in *E. coli*. The pTYB1-PRP and pTYB1-PACAP recombinant plasmids were transformed into electrocompetent *E. coli* strain BL21 harboring the λ DE3 lysogen that carries the T7 RNA polymerase under the control of the lac UV5 promoter. Five milliliters of Luria-Bertani (LB) medium containing 50 µg/ml ampicillin were inoculated with one colony and grown overnight at 37 °C with vigorous shaking. This culture was used to inoculate 1 l LB medium containing 50 µg/ml ampicillin. The expression of the recombinant PRP and PACAP was induced at an optical density (600 nm) of 0.5 by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for 5 h. After induction, the cells were harvested by centrifugation at 5000 g for 20 min and stored frozen until use. The cell pellet was thawed and resuspended in lysis buffer (20 mM Tris-HCl (pH 8), 500 mM NaCl, 1 mM EDTA, 20 µM phenylmethylsulfonyl fluoride). A French press was used to lyse the cells. The expression of the fusion proteins was tested by both 10% SDS-PAGE and western blot analysis. Because the pTYB vectors (New England Biolabs) are not recommended as

control for testing expression, the intact polylinker region apparently causes poor expression and the control plasmid pMYB5 was used. This control plasmid carries the *E. coli* *malE* gene encoding the maltose-binding protein (42 kDa) fused in-frame to the coding region of the intein tag.

To induce cleavage of the PRP and PACAP from the intein fusion protein, the supernatants obtained by lysate centrifugation were incubated overnight at 4 °C with 50 mM cysteine solution (pH 9). Afterward, the supernatants were clarified by centrifugation at 10 000 *g* for 30 min, dialyzed overnight at 4 °C against PBS 1×, and stored at –20 °C until use. The cleavage proteins were checked by both (16.5% T/3% C) Tricine–SDS–PAGE (Schagger & von Jagow 1987) and mass spectrometry (MS). The quantity of cleavage peptide from intein was estimated by comparison with recombinant epidermal growth factor standards (CIGB) in the Tris–Tricine gel.

Protein analysis

Protein concentrations in the clarified lysate of the catfish PRP- and PACAP-transfected *E. coli* BL21 (D3) cells were determined using the BCA Protein Assay kit (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The purity level of the PRP and PACAP cleaved from the intein was estimated as the percentage of each peptide versus total proteins in the appropriate lane of the Tris–Tricine gel stained with Coomassie blue (Bio-Rad). The digital gel images used in these analyses were obtained using a Hewlett–Packard Scanjet Plus. Data were processed with Molecular Analyst software version 1.4.1 (Bio-Rad).

Western blot analysis

To verify the PRP and PACAP expression as an intein-tagged fusion protein, western blot analyses were carried out. The fused PRP and PACAP proteins were electrophoretically fractionated by 10% SDS–PAGE and transferred to a nitrocellulose membrane. After blocking with 5% skim milk (Oxoid) in PBS 1×, the membrane was incubated for 2 h at room temperature with the rabbit serum raised against a peptide derived from the *Bacillus circulans* chitin-binding domain (New England Biolabs). After washing with PBS–T once and with PBS 1× twice, the membrane was incubated with a 1:5000 dilution of anti-rabbit polyclonal antibody HRP conjugate (Amersham Biosciences), as secondary antibody with gently shaking for 1 h at room temperature (RT). The detection was carried out using chemiluminescence with the ECL Western blotting Analysis System (Amersham Biosciences) according to the manufacturer's instructions.

Mass spectrometric analysis of the recombinant PRP and PACAP

Specific bands were incised from Coomassie blue-stained Tris–Tricine gels. These bands were washed with 200 mM NH_4HCO_3 in 50% acetonitrile. After the dehydration of the

gels, PRP was digested with trypsin and PACAP with lysyl endopeptidase in 50 mM NH_4HCO_3 overnight at 37 °C. Derived peptides were eluted and desalted (Gonzalez *et al.* 2003). The ESI–MS spectra were acquired using a QToF-2 (Micromass, Manchester, UK) fitted with a Z-spray nanoflow electrospray ion source operated at 80 °C with a drying gas flow at 50 l/h. The peptides eluted from ZipTips with 60% acetonitrile in 1% formic acid (Millipore, Billerica, MA, USA) were loaded into borosilicate nanoflow tips and submitted to 900 V and 35 V of capillary and cone voltage respectively. To acquire the ESI–MS/MS spectra, the first quadrupole was used to select the precursor ion within a window of ~ 2 Th. Argon was used in the collision chamber at $\sim 3 \times 10^{-2}$ Pa pressure and collision energies between 23 and 45 eV were set to fragment precursor ions. Data acquisition and processing were performed using MassLynx v3.5 (Micromass). Sequence tags were manually extracted and used to identify the proteins by the PepSea program (http://pepsea.protona.com/PA_PeptidePatternForm.html).

Growth-promoting effect of the recombinant PRP and PACAP in fish larvae treated by immersion baths

The growth promotion experiments were performed in *C. gariepinus* larvae 0.020 ± 0.007 g in weight and 1.215 ± 0.128 cm in length ($n=200$), in *O. niloticus* larvae 0.133 ± 0.054 g in weight and 1.537 ± 0.246 cm in length ($n=150$), and in Carp (*Cyprinus* sp.) larvae 0.018 ± 0.005 g in weight and 1.203 ± 0.132 cm in length ($n=450$). Each experiment took place independently. In each assay, the groups were acclimated in 80 l tanks containing running freshwater for 1 week prior to the experiment. The fish were fed to satiation with a basal diet twice a day. Prior to treatment, the tanks were cleaned using a siphon and the amount of water was decreased to 2 l. Then, 10 ml semi-purified recombinant PRP or PACAP, as described above, were added in a quantity equivalent to 400 µg of each peptide. Each treatment was carried out for 90 min without water recirculation. The treatment was repeated three times a week for 4 weeks. In each experiment, one group was included as negative control. This control group received the clarified supernatant of lysis cells transformed with pTYB1 containing equivalent amounts of *E. coli* proteins to the PRP and PACAP treatments. In the experiment with *C. gariepinus* larvae, another control group did not receive any treatment to determine if there was any consequence of the immersion bath procedure in growth performance. Growth-promoting effects were evaluated by the increase in body weight and length. Data were expressed as mean $\pm$ s.d.

Skin pigmentation evaluation

Fish skin pigmentation was determined by visual evaluation and photography at the end of the experiment. A total of 150 carp were analyzed. The presence of red, green, or both pigments was taken into account in this study. Data were

illustrated as the percentage of the total fish that showed red, green, or both skin pigments.

Statistical analysis

The statistical analyses were carried out using GraphPad Prism statistical Software Inc., version 4.00.255 (San Diego, CA, USA). The normality of the data was evaluated using Barlett test (χ^2). Data were also evaluated using one-way ANOVA. Data with normal distribution and equal variances were analyzed using Newman–Keuls multiple comparison test. Data with unequal variances were analyzed by the Kruskal–Wallis test followed by Dunn’s multiple comparison post-test. Treatments were considered to be significantly different if $P < 0.05$.

Results

Isolation of the cDNA precursor encoding *C. gariepinus* PRP/PACAP

A 728 bp cDNA precursor encoding both PRP and PACAP was isolated from the brain of the African catfish (*C. gariepinus*) by RT-PCR using specific primers based on the *I. punctatus* sequence (Fig. 1). The obtained cDNA consisted of the signal peptide from 1 to 60 bp (aa 1–20), a cryptic peptide, PRP region from 250 to 384 bp (aa 84–129), PACAP from 391 to 504 bp (aa 131–169), and the 3′-untranslated region (Fig. 2).

Figure 1 Electrophoresis in 1.5% agarose gel that shows the cDNAs encoding *C. gariepinus* PRP/PACAP, PRP, and PACAP. Lane 1: RT-PCR to amplify the PRP/PACAP cDNA precursor from the brain of the African catfish (*C. gariepinus*). Lane 3: PCR to amplify the cDNA sequence encoding for both signal peptide from PRP/PACAP precursor and PRP with the restriction endonuclease sites to be cloned into pTargetT vector (Promega). Lanes 5 and 7: PCRs to amplify the signal peptide from PRP/PACAP precursor and PACAP sequences respectively, with the restriction endonuclease sites in the correct open reading frame for cloning both into pTargetT vector. Lane 9: positive control RT-PCR using brain total RNA from *C. gariepinus* as template and β -actin-specific primers based on *O. mossambicus* β -actin cDNA. Lane 11: Fragments of the molecular weight marker λ HindIII/EcoRI (HeberBiotec, SA). Lanes 13 and 15: PCRs to amplify the PRP and PACAP cDNAs respectively, with the restriction endonuclease sites necessary for cloning into the pTYB1 bacterial expression vector (New England Biolabs, USA). Lanes 2, 4, 6, 8, 10, 12 and 14: negative control PCRs (without template) with respect to lanes 1, 3, 5, 7, 9, 13 and 15 respectively. For PRP and PACAP cDNA amplifications, the PRP/PACAP cDNA precursor was used as template.

The organization of the *C. gariepinus* PRP/PACAP polypeptide is very similar to other catfish (McRory *et al.* 1995, Small & Nonneman 2001). PACAP is preceded by a dibasic amino acid enzyme-processing site, lysine–arginine, and is followed by a glycine–arginine–arginine-processing site that would yield a 38 amino acid peptide with an amidated C-terminus. Processing at the second amidation site within the PACAP sequence would result in the 27 amino acid PACAP.

Comparison of the *C. gariepinus* PACAP38 sequence with other PACAP sequences in fish showed more than 90% identity (Fig. 3). In general, the deduced amino acid sequence of the *C. gariepinus* PACAP was highly homologous (more than 80% identity) to sequences previously identified from tunicates to mammals (Fig. 3). The PRP sequence was less conserved between fish and higher vertebrates. For example, *C. gariepinus* PRP shared only 35% identity with *Homo sapiens* PRP. The sequence of PRP is only somewhat conserved among closely related species.

Transient expression of the mature PRP and PACAP in mammalian cells

The sequences encoding mature forms of *C. gariepinus* PRP and PACAP were accurately amplified by PCR for cloning into the eukaryotic expression vector pTargetT (Fig. 1). In both cases, the signal peptide corresponding to the *C. gariepinus* PRP/PACAP precursor was used to direct the expressed target proteins to the BHK-21 culture supernatant. The co-transfection of the pCMV-GFP plasmid with each of the DNA vector constructs enabled transfection normalization by microscopic evaluation at the end of the experiment (data not shown).

GH in vitro assays using hemipituitary gland culture

A statistically significant increase in the GH secretion was observed when the pituitary glands were treated for 24 and 48 h with equal supernatant quantities of the PRP- or PACAP-transfected BHK-21 cells ($P < 0.05$) (Table 2). Dose-dependent increases in GH secretion were observed when the pituitary glands were treated with GHRP-6 as positive control up to 5 μ g/pituitary gland (6 nM). At 10 μ g/pituitary gland (12 nM), an inhibition response was observed (Table 2).

Recombinant expression of *C. gariepinus* PRP and PACAP in *E. coli*

The mature forms of the *C. gariepinus* PRP and PACAP were amplified by PCR to be cloned into the pTYB1 expression vector. In both cases, the expected size of 135 bp (PRP) and 114 bp (PACAP) were obtained (Fig. 1). The correct insertion of the target cDNAs into pTYB1 vector was corroborated through restriction endonuclease site and DNA sequencing analysis.

Expression analysis of the PRP and PACAP as intein tags by SDS-PAGE showed bands of the estimated 60 kDa size in the

1	M A K S S R A T L A L L I Y G I L M
1	<u>ATG GCC AAA TCT AGT AGA GCT ACT TTG GCT CTG CTC ATC TAC GGG ATC TTA ATG</u>
19	R Y S A Q C T P I G M G F P N M R L
55	<u>CGC TAC</u> AGC GCC CAA TGC ACA CCC ATC GGA ATG GGC TTC CCC AAT ATG AGG CTA
37	E N D V F G D E G N S L S E L S Y E
109	GAA AAC GAC GTG TTC GGG GAC GAG GGA AAC TCG TTA AGT GAG CTG TCC TAC GAG
55	P D T M S A R S A P A L P E D A Y T
163	CCG GAC ACG <u>ATG</u> AGC GCG CGC AGT GCT CCA GCC CTC CCT GAA GAC GCA TAC ACA
73	L Y Y P P E R R A E T H A D G L L D
217	CTG TAC TAC CCG CCC GAG AGA AGA GCC GAA ACG CAT GCA GAC GGA TTG TTA GAT
91	R A L R D I L V Q L S A R K Y L H S
271	AGA GCC TTG AGG GAC ATC CTG GTT CAG TTA TCA GCC CGA AAA TAT CTG CAT TCT
109	L T A V R V G E E E E D E E D S E P
325	CTG ACG GCA GTT CGC GTA GGT GAG GAA GAA GAG GAT GAA GAG GAC TCG GAG CCA
127	L S K R H S D G I F T D S Y S R Y R
379	CTG TCG AAG CGC CAC TCG GAC GGC ATT TTC ACG GAC AGC TAC AGC CGC TAC CGG
145	K Q M A V K K Y L A A V L G R R Y R
433	AAA CAA ATG GCC GTA AAA AAA TAC CTT GCA GCA GTG CTG GGA AGA AGG TAC AGA
163	Q R F R N K G R R F A Y L
487	CAG AGG TTT AGA AAC AAA GGA CGC CGC TTT GCT TAT TTG <u>TAG</u> CGGATAGGAAGAAAAGG 545
546	AAAGAAAGAAAAAACGCGAGAGAGAGAGAGAGAGAGAGAGAAATAGAGCAACTGCCCTCCCTTGTGTCCATTC 617
618	AATCATACAGTCAAGAAGTCTGGTATCTAACTTAACACTGAGCAGTCAGTCGGTGGATCTCGCCTGTGTTCTT 689
690	TTAAACATGTATTTTATGTACGAAGTCAAGCC <u>ATTAAAG</u>

Figure 2 Nucleotide sequence and deduced amino acid sequence of the *C. gariepinus* PRP/PACAP cDNA precursor (GenBank accession number EF524513). The sequence encoding PRP is shaded in gray and the sequence encoding PACAP is in bold and shaded in gray. The signal peptide coding sequence is shown by a single underline. The ATG corresponds to the alternative translation initiation start site, the triplet coding for the stop codon (TGA), and the putative polyadenylation signal (ATTAAA) are in bold and double underlined.

lanes corresponding to lysate supernatants of the *E. coli* BL21 (DE3) transformed with PRP and PACAP (Fig. 4A). The maximum levels of expression of the target proteins were observed 5 h after IPTG addition at 28 °C. Expression levels of target proteins fused to intein were identified using rabbit serum raised against the chitin-binding domain that recognizes the intein tag (Fig. 4B). In the lanes corresponding to lysate supernatants of the *E. coli* BL21 (DE3) transformed with PRP and PACAP, bands of the expected 60 kDa size were identified. This band was absent in the control lysate of *E. coli* BL21 (DE3) (Fig. 4B). The maltose-binding protein fused in-frame to the coding region of the intein tag was present (data not shown). The non-specific band above 60 kDa was also recognized with rabbit serum against chitin-binding domain in all samples, including negative controls.

Using the inducible self-cleavage activity of the intein in the presence of thiols (cysteine), an efficient release of the target proteins from the intein tag was obtained. The cleavage proteins showed the estimated sizes of the ~5 kDa in the Tris–Tricine gel and were obtained ~50% pure (Fig. 4C). Both peptides were obtained at about 3 mg per l of culture medium. MS analysis of the cleavage proteins (Fig. 4C) demonstrated that these correspond to PRP and PACAP from catfish.

Protein identifications by MS

Signals at m/z 659·62 (2+) and 514·25 (2+) were observed after tryptic digestion of PRP and lysyl endopeptidase treatment of PACAP respectively. ESI-MS/MS analyses of these peptides are shown in Fig. 5A and B.

Fish	PRP	S	I (%)	PACAP	S	I (%)
C. gariepinus	HADGLLDRLALRDILVQLSARKYLHSLTAVRVGEEE--EDEEDSEPLS	45		HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRFRNK	38	
C. macrocephalus	HADGLLDRLALRDILVQLSARKYLHSLTAVRVGEEE--EDEEDSEPLS	45	100	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRFRNK	38	100
I. punctatus	HADGLLDRLALRDILVQLSARKYLHSLTAVRVGEEE--EDEEDSEPLS	45	100	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRFRNK	38	100
G. morhua	HAEEELDRALREILGQLTARQYLHSLMTIRVGEET--SMEDESEPLS	45	66	HSDGIFTDSYSRHRKQMAVKKYLA AVLGRYRQRVRNK	38	94
T. nigroviridis	HAEGELDRALREILGQLTARHYRHFMTVR-G----SAE-ESEPLS	40	55	HSDGIFTDSYSRYRKQMAVQKYLA AVLGRYRQRVRNK	38	94
D. rerio	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGST--TEDDNEPLS	45	60	HSDGIFTDIYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	92
O. mykiss	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGST--MEDDSEPLS	45		HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	92
C. carpio				HSDGIFTDIYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	92
C. idella	HADGLLDRLALRDILVQLSARKYLHSLMAVRVGGGSS--EEDSEPLS	45	82	HSDGIFTDIYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	92
Tunicate						
H. roretzi				HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNE	38	92
Amphibians						
X. laevis	HADELLNKVYRNVLGHLSARKYLHSLMAQLRTVSS--SLEDESEPLS	46	55	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
R. ridibunda	--DFAFDNNPIGIGNPASVFDDMYSFYYPAEKSAVSSSLEDDESEPLS	45	20	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
Birds						
O. aries	--HGILDKAYRKVLQDLSARRYLQTLMAKLGGLTPGGGADDDSEPLS	45	37	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
A. platyrhynchos	HADGIFNKAYRKLLGQLSARKYLHSLMAKRVGGSSG-LGDEAEPLT	46	51	HIDGIFTDSFSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	84
G. gallus	--DFALRAGAPGGGPRPRWRGCTALYYPGKSGASSGLGDEAEPLS	45	11	HIDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	86
Mammalian						
C. familiaris	--QGILNKAYRKVLQDLSARKYLQTLVAKGLGGLNGLGGGAEDDSEPLS	45	51	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
O. cuniculus	--LGILNKAYRKVLQDLSARKYLQTLMAKGMENLGGGPEDDLEPLS	45	51	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
S. scrofa	--HGILNKAYRKVLQDLSARKYLQTLMAKSVGGNLDGGAEDDSEPLS	45	53	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
M. musculus	--HEILNEAYRKVLQDLSARKYLQSVVARGAGENLGGSAVDPAPLT	45	37	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
R. norvegicus	--HEILNEAYRKVLQDLSARKYLQSMVARGMGENLAAA VDDRAPLT	45	35	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
H. sapiens	--HGILNEAYRKVLQDLSAGKHLQSLVARGVGGSLGGGAGDDAEPLS	45	35	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
M. fascicularis	--HGILNKAYRKVLQDLSARKYLQSLVARGVGGSLGGGVEDEAEPLS	45	55	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
P. troglodytes	--HGILNKAYRKVLQDLSARKYLQSLVARGVGGSLGGGAEDDAEPLS	45	53	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
B. taurus	--HGILNKAYRKVLQDPSARRSPADAHGQGLGWDPGGSADDDSEPLS	45	24	HSDGIFTDSYSRYRKQMAVKKYLA AVLGRYRQRVRNK	38	89
	:	.	:	*	*****	.*:*****:*****:.*:* :..

Figure 3 Comparison of PRP and PACAP sequences among species. Sequence analysis comparison was made by CLUSTALW analysis (<http://www.ebi.ac.uk/Tools/clustalw>) among PRP/PACAP amino acid sequences reported for *Clarias macrocephalus* (CAA55684), *Ictalurus punctatus* (AAK66970), *Gadus morhua* (AAZ85701), *Tetraodon nigroviridis* (CAG12289), *Danio rerio* (AAH83516), *Oncorhynchus mykiss* (AAK28557), *Cyprinus carpio* (BAD01118), *Ctenopharyngodon idella* (EF592488), *Halocynthia roretzi* (BAD01117), *Xenopus laevis* (AAD56956), *Rana ridibunda* (AAF74571), *Ovis aries* (NP_001009776), *Anas platyrhynchos* (ABE01122), *Gallus gallus* (AAX56089), *Canis familiaris* (XP_849191), *Oryctolagus cuniculus* (FAD78945), *Sus scrofa* (AAD12780), *Mus musculus* (BAC37673), *Rattus norvegicus* (NP_058685), *Homo sapiens* (NP_001108), *Macaca fascicularis* (AAW59436), *Pan troglodytes* (XP_001147286), *Bos taurus* (NP_001040020), and that obtained from *C. gariepinus* (EF524513). The GenBank accession numbers of the sequences are indicated in the parentheses. *Indicates the conserved amino acids. :, Indicates similar amino acid. ., Indicates different amino acid. I, identity percentage among species; S, peptide size in amino acid.

Both spectra were manually analyzed and very reliable extracted sequences were used to confirm the identity of the characterized proteins. In the sequence databases, the extracted peptide sequences corresponded to the N-terminal end of PACAP and PRP polypeptides.

Table 3 shows all signals obtained and their respective sequences assigned by ESI-MS/MS analysis. Differences between *m/z* observed and calculated were associated with the internal error of the mass spectrometer (below 50 ppm). The chemical modification observed in aspartic acid was generated by *E. coli* post-translation modifications.

Growth-promoting activity of the recombinant C. gariepinus PRP and PACAP in fish larvae

Experiments were performed to evaluate the biological effect of the recombinant *C. gariepinus* PRP and PACAP on the growth rate of catfish (*C. gariepinus*), tilapia (*O. niloticus*), and carp (*C. carpio*). The effect of these peptides on fish growth was determined by measuring the increase in body weight and length.

Growth-promoting activity in C. gariepinus larvae

The PRP- and PACAP-treated groups showed a significant increase in body weight and length compared with the

control and BL21 groups, 8 days after treatment ($P<0.01$). After 15 days, both weight and length increments, when compared with the BL21 and control groups, were highly significant ($P<0.001$). Statistically significant differences

Table 2 Effects of the recombinant *C. gariepinus* pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP-related peptide (PRP) expressed in baby hamster kidney (BHK)-21 cells on growth hormone (GH) secretion by cultured tilapia pituitary cells. Data represent arithmetic mean of the tilapia GH $\pm$ s.d.

Treatment ^a	GH concentration (ng/ml)	
	24 h	48 h
pCMV-SP-PRP	15.01 $\pm$ 0.93 ^b	22.79 $\pm$ 5.21 ^b
pCMV-SP-PACAP	14.97 $\pm$ 1.89 ^b	22.23 $\pm$ 5.78 ^b
pTarget	8.02 $\pm$ 0.81	12.60 $\pm$ 2.36
Negative control	9.21 $\pm$ 1.79	10.67 $\pm$ 2.84
GHRP-6 (3 nM)	— ^d	12.37 $\pm$ 1.25
GHRP-6 (6 nM)	— ^d	22.19 $\pm$ 3.76 ^c
GHRP-6 (12 nM)	— ^d	14.67 $\pm$ 2.63

^aTreatments were tested in duplicate, with three to four fish pituitaries each test.
^bStatistical differences with respect to the negative and pTarget controls ($P<0.05$).
^cStatistical differences with respect to negative control ($P<0.05$).
^dNot measured.

Figure 4 Recombinant expression in *E. coli*, immune identification, and semi-purification of the *C. gariepinus* PRP and PACAP. (A and B) Glycine-SDS-PAGE and western blotting respectively to analyze the expression of the PRP and PACAP in *E. coli*. Protein expressions were performed at 28 °C for 5 h. Lanes 1 and 2: lysis supernatant and pellet respectively from *E. coli* BL21 (DE3) transformed with pTYB1-PRP expression vector. Lanes 3 and 4: lysis supernatant and pellet respectively from *E. coli* BL21 (DE3) transformed with pTYB1-PACAP. MW, broad-range protein marker (Bio-Rad). Lanes 5 and 6: lysis supernatant and pellet from *E. coli* BL21 (DE3) transformed with pTYB1. (C) Tricine-SDS-PAGE analysis from PRP and PACAP semi-purification. The target proteins were obtained ~50% pure. Lanes 1 and 2: lysis supernatants from *E. coli* BL21 (DE3) transformed with pTYB1-PRP and pTYB1-PACAP respectively treated with 0.5 mM of cysteine solution pH 9 overnight at 4 °C, clarified by centrifugation at 10 000 *g* for 30 min, and dialyzed overnight at 4 °C against PBS 1×. The identity of the shown semi-purified peptides was demonstrated by mass spectrometry (Fig. 5). Arrows denote the target proteins.

($P < 0.05$) in body weight increment in the PACAP-treated group, when compared with PRP treated groups, were also observed at 21 days (Fig. 6A and B). No statistical difference in increase in length was observed between the PRP and PACAP groups at that time (Fig. 6B).

No statistical difference in body weight increase and length was observed in the BL21 group when compared with the control group in the experiment (Fig. 6A and B).

Growth-promoting activity in *O. niloticus* larvae

Eight days after the start of the experiment, the PRP- and PACAP-treated groups showed a significant increase in body weight and length compared with the control group ($P < 0.05$). The difference in body weight increment between the PACAP-treated group and the BL21 group was highly significant ($P < 0.001$) at days 15 and 21, and a statistically significant difference was also observed between PACAP- and PRP-treated groups ($P < 0.05$) during these times (Fig. 7A and B). No statistical difference in increase in length was observed between the PRP and PACAP groups during the experiment (Fig. 7B).

Growth-promoting activity in *C. carpio* larvae

The PACAP-treated group showed a statistically significant increase in body weight and length compared with the BL21 group, 8 days after the start of the experiment ($P < 0.01$; Fig. 8). Statistically significant increase in the body weight ($P < 0.05$) and length ($P < 0.01$) between the PRP- and BL21-treated groups were also observed at this time. At day 15, the differences in the body weight of the PACAP-treated groups, when compared with the BL21 group were highly significant ($P < 0.001$). At day 27, statistically significant differences in body weight increase were observed between the PACAP- and PRP-treated group ($P < 0.05$). Statistically significant differences were also observed in the increase in length in the PRP- and PACAP-treated groups when compared with the BL21 group 8 days ($P < 0.01$) and 15 days ($P < 0.001$) after the start of the experiment, but no statistically significant difference was detected in the length average between the PRP and PACAP groups during the experiment (Fig. 8).

Figure 5 ESI-MS/MS spectra of the N-terminal peptides for (A) PACAP *m/z* 659.62 (3+) and (B) PRP *m/z* 514.25 (2+) polypeptides.

Table 3 Peptide sequences identified by mass spectrometry of the recombinant pituitary adenylate cyclase-activating polypeptide (PACAP)-related peptide (PRP) and PACAP expressed in *E. coli*

	Peptide sequence	Mass/charge ratio ^a		Charge
		Observed	Calculated	
Peptides				
	PACAP			
	¹ MHSD ^b GIFTDSYSRYRK ¹⁶	659·62	659·64	3
	²² YLA AVLGR ²⁹	431·76	431·75	2
PRP	¹ MHADGLLDR ⁹	514·25	514·25	2
	¹³ DILVQLSAR ²¹	507·80	507·80	2
	³² VGEEEEDEEDSEPLS ⁴⁶	846·84	846·84	2

^aMass accuracy determination was below 50 ppm.
^bD aspartic acid expected, mass difference corresponds to methyl aspartic acid.

The carp larvae treated with PACAP showed precocity in their skin pigmentation when compared with the BL21-treated group. At 27 days, the PACAP-treated group had red pigmentation on 24% of their total body surfaces, whereas the PRP- and BL21-treated fish showed only 5% and 9% respectively (Table 4).

Discussion

We have isolated from African catfish (*C. gariepinus*) the cDNA encoding a single precursor containing both PRP and PACAP. The organization of the precursor is similar to that described in other non-mammals: signal peptide, cryptic peptide, PRP, PACAP, and 3'-UTR (Adams *et al.* 2002). The deduced amino acid sequence of the *C. gariepinus* PACAP is

89% identical to the *H. sapiens* sequence, whereas the PRP is only 35% identical to human PRP. The high conservation of the PACAP peptide sequence throughout evolution points to an important role for this molecule (Vaudry *et al.* 2000). This finding is in agreement with the multiple biological functions described for this peptide, which include hypophysiotropic, neurotransmitter, neuromodulator, and vasoregulatory activities (Carlsson *et al.* 1996, Wong *et al.* 1998, Shioda *et al.* 2006). The low conservation of the PRP sequence suggests that its function is less critical and could even be lost during evolution. This hypothesis may be supported by phylogenetic studies that appear to indicate the loss of the PRP receptor in the mammalian genome (Lee *et al.* 2007).
In the present work, using hemipituitary gland culture from tilapia (*O. niloticus*), we established that *C. gariepinus* PRP and PACAP are able to stimulate GH secretion *in vitro*.

Figure 6 Growth promotion experiment in *C. gariepinus* larvae ($n=200$) immersed in the *E. coli*-derived PACAP and PRP at a dose of 200 $\mu\text{g/l}$ of water. (A) Effect on body weight over 21-day period. The picture was taken at 21 days of treatment. (B) Effect on length over 15-day period. Samples of 25 animals were weighted and sized at 0, 8, and 15 days. Samples of 80 animals were weighted at 21 days. Control group: did not receive any treatment. BL21 group: received lysis supernatant from *E. coli* BL21 (D3) transformed with pTYB1 vector. PRP group: received lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PRP vector. PACAP group: received lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PACAP vector. Newman-Keuls multiple comparison test was used for body weight comparison among groups at days 0 and 8; the letter 'b' indicates $P<0.01$. Kruskal-Wallis test followed by Dunn's multiple comparison post-test was used at days 15 and 21; the letter 'b' indicates $P<0.001$ and 'c' $P<0.001$ with respect to control and BL21 groups and $P<0.05$ with respect to PRP group. In the case of length, Newman-Keuls multiple comparison test was used at days 0, 8, and 15; the letter 'b' indicates $P<0.01$ at 8 days and $P<0.001$ at 15 days and 'a' $P>0.05$. Bars indicate s.d.

Figure 7 Growth promotion experiment in *O. niloticus* larvae ($n=150$) immersed in the *E. coli*-derived PACAP and PRP at a dose of 200 $\mu\text{g/l}$ of water. (A) Effect on body weight over 21-day period. The picture was taken at 30 days after the last immersion bath (carried out at 21 days after the start of the experiment). (B) Effect on length over 15-day period. Samples of 25 animals were weighted and sized at 0, 8, and 15 days. Samples of 50 animals were weighted at 21 days. BL21 group: received lysis supernatant from *E. coli* BL21 (D3) transformed with pTYB1 vector. PRP group: received lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PRP vector. PACAP group: received lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PACAP vector. Newman-Keuls multiple comparison test was used for body weight comparison among groups at days 0 and 15; the letter 'b' indicates $P<0.05$, and 'c' $P<0.001$ with respect to BL21 group and $P<0.05$ with respect to PRP group. Kruskal-Wallis test followed by Dunn's multiple comparison post-test was used at days 8 and 21. In these cases, the letter 'b' indicates $P<0.05$ at day 8 and $P<0.01$ at day 21 and 'c' $P<0.001$ at day 21 with respect to BL21 group and $P<0.05$ with respect to PRP group. In the case of length, Newman-Keuls multiple comparison test was used at days 0 and 15; the letter 'b' indicates $P<0.01$ between PRP and BL21 groups and $P<0.001$ between PACAP and BL21 groups. Kruskal-Wallis test followed by Dunn's multiple comparison post-test was used at day 8; the letter 'b' indicates $P<0.05$ and 'a' $P>0.05$. Bars indicate s.d.

Dose-dependent increases in GH secretion were observed when the pituitary glands were treated with GHRP-6 as positive control. Partial desensitization can occur at high concentrations of GHRP-6 (12 nM) probably by negative feedback signals (Giustina & Veldhuis 1998).

The PACAP effects in GH release have been demonstrated *in vitro* in some species of mammals (Hart *et al.* 1992, Jarry *et al.* 1992, Hashizume *et al.* 1994, Sawangjaroen & Curlewis 1994, Chiodera *et al.* 1996, Sawangjaroen *et al.* 1997, Martinez-Fuentes *et al.* 1998), birds (Peeters *et al.* 1998),

Figure 8 Growth promotion experiment in *C. carpio* larvae ($n=450$) immersed in the *E. coli*-derived PACAP and PRP at a dose of 200 $\mu\text{g/l}$ of water. (A) Effect on body weight over 27-day period. The picture was taken at 27 days of treatment. (B) Effect on length over 15-day period. Samples of 25 animals were weighted and sized at 0, 8, and 15 days. Sample of 150 animals were weighted at 27 days. BL21 group: received lysis supernatant from *E. coli* BL21 (D3) transformed with pTYB1 vector. PRP group: received lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PRP vector. PACAP group: received lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PACAP vector. Newman-Keuls multiple comparison test was used for body weight comparison among groups at days 0 and 15; the letter 'b' indicates $P<0.05$ between PRP and BL21 groups and $P<0.001$ between PACAP and BL21 groups. Kruskal-Wallis test followed by Dunn's multiple comparison post-test was used at days 8 and 27; the letter 'b' indicates $P<0.05$ between PRP and BL21 groups, $P<0.01$ between PACAP and BL21 groups at day 8, and $P<0.01$ between PRP and BL21 groups at day 27, and the letter 'c' indicates $P<0.001$ between PACAP and BL21 groups and $P<0.05$ between PRP and PACAP groups. In the case of length, Newman-Keuls multiple comparison test was used at days 0, 8, and 15; the letter 'b' indicates $P<0.01$ at day 8 and $P<0.001$ at day 27 and 'a' $P>0.05$. Bars indicate s.d.

Table 4 Skin color development in carp larvae (*Cyprinus* sp.) treated by immersion with recombinant pituitary adenylate cyclase-activating polypeptide (PACAP) or PACAP-related peptide peptides expressed in *E. coli* at a dose of 200 µg/l of water. Skin pigmentation was determined by visual evaluation and photography at day 27 from treatment. Data are given as the percentage of the total fish showing red, green, or both (red/green) skin pigments

Treatment	Skin pigmentation (%) ^a		
	Red	Red/green	Green
BL21	4·2	31·7	64·1
PRP	8·8	36·8	54·4
PACAP	24·2	48·4	27·3

^aHundred and fifty animals were characterized in each treated group.

amphibians (Gracia-Navarro *et al.* 1992, Yon *et al.* 1993), and fish (Parker *et al.* 1997, Montero *et al.* 1998, Wong *et al.* 1998, Xiao *et al.* 2002, Wong *et al.* 2005). Studies to investigate the effect of PACAP on GH secretion in mammals showed contradictory results. Some data suggest that PACAP stimulates GH release in rat (Hart *et al.* 1992, Jarry *et al.* 1992) and sheep (Sawangaroen *et al.* 1997). However, in humans, i.v. administration of PACAP does not modify plasma GH levels (Chiodera *et al.* 1996). Also, PACAP is less potent than GHRH in stimulating GH release from somatotrophic adenoma cells in primary culture (Adams *et al.* 1994). These findings suggest that, in mammals, the PACAP effect on GH secretion may vary from species to species. In fish, PRP exerts a modest GH stimulatory effect *in vitro* in trout (*Oncorhynchus mykiss*) (Luo *et al.* 1990), carp (*C. carpio*) (Vaughan *et al.* 1992), tilapia hybrid *O. niloticus* × *Oreochromis aureus* (Melamed *et al.* 1995), and salmon (*Oncorhynchus nerka*) (Parker *et al.* 1997). By contrast, PACAP provokes a robust stimulation of GH release from goldfish (*Carassius auratus*) (Wong *et al.* 1998), eel (Montero *et al.* 1998), and grass carp (*Ctenopharyngodon idellus*) (Sze *et al.* 2007) pituitary cells.

In this study, *C. gariepinus* PRP and PACAP were able to stimulate GH secretion by the tilapia pituitary gland *in vitro*. This observation suggests that both peptides are involved in the neuroendocrine control of the somatotroph cell activity in teleost fish. This positive response was observed for *in vitro* static hemipituitary gland culture even though these always lack some of the regulatory factors present *in vivo*.

There are few studies about the effect of PACAP on GH secretion *in vivo*. It is known that this peptide increases the GH levels in rat and bovine plasma *in vivo* (Jarry *et al.* 1992, Radcliff *et al.* 2001), whereas in ewes and humans it does not produce this effect (Sawangaroen & Curlewis 1994, Chiodera *et al.* 1996). To date, there have been no previous *in vivo* studies that show the PACAP and PRP functions with regard to GH stimulation and growth regulation in fish. In this work, the biological effect of the *C. gariepinus* recombinant PRP and PACAP, administered by immersion baths, on the growth rate of *C. gariepinus*, *O. niloticus*, and *C. carpio* larvae was evaluated.

We have reported for the first time an efficient recombinant expression of fish PRP and PACAP in *E. coli* and demonstrated that the growth rate of fish is enhanced by both PRP and PACAP administrations.

The immersion bath method successfully used for our studies requires little manipulation and causes minimum stress to fish during treatment (Nakanishi & Ototake 1997). There is evidence suggesting that the gill pillar cells are a possible entry site for some molecules when fish are treated by immersion bath (Sherwood & Harvey 1986, Moriyama & Kawauchi 1990, Nakanishi & Ototake 1997). For example, in goldfish, gonadotropin-releasing hormone appeared quickly in the plasma following its application to gills (Sherwood & Harvey 1986). In our laboratory, we have employed the immersion bath technique to study the effects of nutritional supplements and growth factors on growth control and the immune system in fish (Martinez *et al.* 2006, Acosta *et al.* 2007, Carpio *et al.* 2007).

In this study, as early as 1 week after the application of the *E. coli*-derived PRP and PACAP, statistically significant increase in body weight and length were obtained in the treated fish larvae when compared with control groups. This result is potentially important to the fish farming industry considering that the highest levels of fish losses occur during the early developmental stages.

We have also observed in the carp larvae treated with recombinant *C. gariepinus* PACAP a precocity in the skin pigmentation change. This observation is consistent with recent studies in *Xenopus laevis* that demonstrated a physiological function of the PACAP/VIP family of peptides in the regulation of the melanotroph cell activity during the process of skin color adaptation (Kidane *et al.* 2007). Additional studies would be required to elucidate the molecular action of PACAP in skin color development in fish.

The results presented here also confirmed that the PRP and PACAP biological functions are well conserved in fish, since *C. gariepinus* PRP and PACAP stimulate growth in *O. niloticus* and *C. carpio* larvae as well as in *C. gariepinus* larvae. In accord with previous *in vitro* studies suggesting that fish PACAP has greater ability than PRP to stimulate secretion of GH in fish (Parker *et al.* 1997), we have demonstrated for the first time that PACAP rather than PRP plays a physiological role in the growth control in teleost fish.

The minor effect of the recombinant *C. gariepinus* PRP on growth performance in tilapia and carp larvae could be attributed to species specificity, inasmuch as the structure of PRP has been less conserved than other hypophysiotropic neuropeptides. In addition, using sequence alignment and phylogenetic analysis, Tam *et al.* (2007) demonstrated that in zebrafish, fugu, tetraodon, stickleback, and goldfish, two distinct PRPs (salmon like and catfish like) could be discerned. The two peptides could also differ in their biological activity. The higher growth-promoting effect observed in *C. gariepinus* larvae treated with recombinant *C. gariepinus* PACAP, when compared with PRP, suggests an earlier role for PACAP, than PRP, in the growth control in teleost fish.

Growth in fish is regulated by the brain neuroendocrine-GH-insulin-like growth factor axis (Peter & Marchant 1995). GH plays an important role as metabolic regulator, especially in stimulating lipid mobilization and protein increase, both of which encourage growth in fish (Hernandez Llorente *et al.* 2004). In the present study, we observed an increase in GH secretion in fish pituitary glands treated with both PACAP and PRP, when compared with control groups. Taking into account our results and the well-known effects of GH in vertebrate growth, the action of PRP and PACAP in fish growth enhancement might be mediated by GH. However, it remains to be elucidated whether the action of these neuropeptides on fish growth is achieved solely through GH stimulation or in conjunction with another, more direct mechanism affecting cell growth. In previous investigations, it was demonstrated that PACAP exerts direct trophic effects on rat cerebellar cortex during development (Vaudry *et al.* 1999). Certainly, further studies are necessary to investigate the possible trophic action of PACAP in teleost fish in cells other than somatotrophs.

As a matter of fact, PACAP has been implicated in a broad range of biological processes in higher vertebrates, including reproduction, development, growth, cardiovascular, respiratory, and digestive functions, immune responses, and circadian rhythms (Vaudry *et al.* 2000). However, there are few *in vivo* studies showing its biological activities in fish (Matsuda *et al.* 2005a,b, Sherwood & Wu 2005, Matsuda & Maruyama 2007). Until now, there have been no *in vivo* studies showing PRP and PACAP activity in fish growth enhancement. Recently, we have also demonstrated PACAP action as a regulator of the teleostean immune system, together with its physiological role in growth control in fish (Carpio & Lugo unpublished observations). Our studies thus far identify PACAP as a prominent target with the potential to stimulate fish growth in aquaculture. In this direction, further research is needed to fully evaluate its potential as a novel fish biotechnology product.

In summary, the results obtained here by *in vitro* and *in vivo* administration of recombinant PRP and PACAP to fish showed for the first time the biological role of these neuropeptides in fish growth and development. This finding contributes toward our understanding of the neuroendocrine axis proposed to explain the hypothalamic regulation of growth in non-mammalian vertebrates. This finding also might lead to a powerful new biotechnology tool to further develop modern fish farming.

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