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A Homolog of Mammalian PRL-Releasing Peptide (Fish Arginyl-Phenylalanyl-Amide Peptide) Is a Major Hypothalamic Peptide of PRL Release in Teleost Fish

SHUNSUKE MORIYAMA, TOSHIHIRO ITO, AKIYOSHI TAKAHASHI, MASAFUMI AMANO, STACIA A. SOWER, TETSUYA HIRANO, KUNIO YAMAMORI, AND HIROSHI KAWAUCHI

Kitasato University School of Fisheries Sciences (S.M., T.I., A.T., M.A., K.Y., H.K.), Sanriku, Iwate 022-0101, Japan; Department of Biochemistry and Molecular Biology, University of New Hampshire (S.A.S.), Durham, New Hampshire 03824-3544; and Hawaii Institute of Marine Biology, University of Hawaii (T.H.), Kaneohe, Hawaii 96744

Two PRL-releasing peptides (PrRP20 and PrRP31) were recently identified from mammalian hypothalamus by an orphan receptor strategy, and a C-terminal RF (arginyl-phenylalamyl-) amide peptide (RFa), structurally related to mammalian PrRP, was also identified from the brain of the Japanese crucian carp (C-RFa) by an intestine-contracting assay. However, to date there have been no reported studies that have examined the PRL-releasing effects of RFa in fish. In the present study we determined the cDNA, primary structure, and function of a homolog of the mammalian PrRP20 in the chum salmon, *Oncorhynchus keta*. An RFa cDNA encoding a preprohormone of 155 amino acids was cloned from the hypothalamus of chum salmon by 3′- and 5′-rapid amplification of cDNA ends. A native RFa was purified from an acid extract of salmon hypothalami by a Sep-Pak C_{18} cartridge, affinity chromatography using anti-synthetic C-RFa, and reverse phase HPLC on an ODS-120T column. The salmon RFa proved to be identical with C-RFa on the basis of elution position on reverse phase HPLC. Immunocytochemical staining in rainbow trout, *Oncorhynchus mykiss*, revealed that C-RFa-immunoreactive cell bodies were located in the posterior part of hypothalamus and C-RFa-immunoreactive fibers were abundant from the hypothalamus to the ventral telencephalon. A small number of immunoreactive fibers were projected to the pituitary and terminated close to the PRL cells in the rostral pars distalis and to the somatolactin (SL) cells in the pars intermedia. The hypophysiotropic effects of the fish homolog were determined on the release of PRL, SL, and GH from the pituitary of the rainbow trout. Plasma PRL and SL levels were increased at 3 and 9 h, respectively, after ip injection of the synthetic C-RFa into the rainbow trout at doses of 50 and 500 ng/g body weight. In contrast, plasma GH levels were decreased after 1 h at 500 ng/g body weight. Perifusion of the trout pituitaries with synthetic C-RFa at concentrations of 10 pM to 100 nM demonstrated maximum PRL release at 100 pM and maximum SL release at 10 and 100 nM. However, GH release was not affected. These data are the first to demonstrate that a homolog of mammalian PrRP (fish RFa) is a major hypothalamic peptide of PRL release in teleost fish. (*Endocrinology* 143: 2071–2079, 2002)

RELEASE OF ANTERIOR pituitary hormones such as GH, ACTH, gonadotropins, and TSH is stimulated by specific hypothalamic peptide hormones, whereas PRL secretion has been thought to be regulated primarily by dopaminergic inhibition (1) until quite recently. Hinuma *et al.* (2) identified a novel hypothalamic peptide with a potent PRL-releasing activity from bovine hypothalamus extract as a ligand of an orphan G protein-coupled receptor (hGR3), which was predominantly expressed in the anterior pituitary. This neuropeptide, termed PrRP, possesses two molecular forms, a 31-amino acid peptide (PrRP31) and the C-terminal 20 resides (PrRP20), which belong to the so-called arginyl-phenylalanyl-amide peptides (RFa). However, there are contradictory data on PRL-releasing effects that have been reported (3, 4), including release of oxytocin (3), ACTH (5), and gonadotropin (6) and inhibitory effects on GH release (7).

At about the same time as the finding of mammalian PrRP, Fujimoto *et al.* (8) isolated a novel peptide from the brain of the Japanese crucian carp, *Carassius auratus langsdorfii*, using the fish intestine-contracting assay. Sequence comparison revealed that the carp RFa (C-RFa) is a homolog of bovine PrRP20 with 65% identity. This coincidence of the two findings suggested that the RFa gene may have been conserved throughout vertebrates. This then resulted in the present study, which was to determine the structure and function of this novel peptide, including PRL-releasing activity, in teleost fish. To date, a specific hypothalamic factor for PRL release has not been clearly demonstrated, although it has been reported that GnRH (9), TRH (10), and E2 (11) stimulate PRL release in fish. To accomplish our objective, we chose the salmon family of fish to determine whether this novel peptide is indeed a hypothalamic peptide for PRL release. As no method is available to measure the activity of this new peptide in carp, we decided to examine the PRL-releasing activity of this peptide in salmon, in which PRL RIA has been developed. This required us to first identify the structure of PrRP in salmon and then to determine its distribution in the brain as well as its function.

In teleost fish, PRL along with GH and a new hormone, somatolactin (SL), from the pars intermedia comprise the pituitary hormone family that is similar in structure and gene organization (12). Thus, these hormones are thought to have evolved from a common ancestral gene by duplication and

Abbreviations: BW, Body weight; C-RFa, carp RF (arginyl-phenylalamyl-) amide peptide; PrRP, PRL-releasing peptide; RACE, rapid amplification of cDNA ends; RFa, RF (arginyl-phenylalamyl-) amide peptide; SL, somatolactin; TFA, trifluoroacetic acid.

subsequent divergence. PRL plays an important role in fresh water adaptation in euryhaline teleosts (13, 14) and is also implicated in other biological actions, such as reproduction (15), behavior, and metabolism (16). GH stimulates somatic growth in teleost fish as well as in other vertebrates and is also involved in seawater adaptation (17, 18). SL has been suggested to be involved in reproduction (19, 20), calcium regulation (21), stress response (22), adaptation to the environment (23, 24), and acid/base balance (25, 26). Therefore, the present study compared hypophysiotropic effects of RFa on the release of three evolutionary related hormones, PRL, GH, and SL, in the rainbow trout both *in vivo* and *in vitro*.

Materials and Methods

Fish

Hypothalami were excised from the brains of mature female chum salmon, *Oncorhynchus keta*, ascending the Tsugaruishi River (Iwate Prefecture, Japan). They were frozen immediately in liquid nitrogen and stored at −80 C until use. Immature rainbow trout, *O. mykiss* [100 g mean body weight (BW)], were raised in indoor freshwater tanks under natural water temperature (12−13 C) and photoperiod at the School of Fisheries Sciences, Kitasato University. Immature rainbow trout (300 g mean BW) were also raised in indoor freshwater tanks at 10 C under natural photoperiod at the University of New Hampshire.

Cloning of salmon RFa cDNA

Total RNA was extracted from 150 mg chum salmon hypothalami using Isogen (Nippon Gene, Tokyo) according to the manufacturer's protocol. The concentration of total RNA was estimated by measuring the absorbance at 260 nm (conversion factor: 1 OD = 40 μg RNA/ml), and the purity was determined from the ratio of absorbance at 260/280 nm. First-strand cDNA was reverse transcribed from total RNA using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. Two degenerate sense primers and the *Not*I adapter primer were used to clone the 3′ partial region of putative chum salmon RFa cDNA. These sense primers were designed based on the conserved regions of mammalian PrRPs (27) and C-RFa (28) as follows: sRF-1, 5′-CCi(TG)(TC)iTGGTA(TC)(GA)(TC)-3′; and sRF-2, 5′-Ci(GA)TiGGi(CA)GiTT(TC)GGi(CA)-3′.

During PCR, 50 μl reaction mixture [1 μl first strand cDNA (template), 2 μl each of sense and *Not*I adapter primers (final concentration, 0.4 μM), 4 μl nucleotide mix (0.2 mM), and 5 μl 10× PCR buffer (final concentrations of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl, 1.25 U Gold-Taq DNA polymerase (PE Applied Biosystems, Foster City, CA)] were subjected to 30 cycles of amplification by PCR. After activation of *Taq* at 94 C for 15 min, each cycle consisted of 1-min denaturation at 94 C, 1-min primer annealing at 40 C, and 1-min, 30-sec primer extension at 72 C. The final extension was 7 min at 72 C.

The 5′-end of salmon RFa cDNA was amplified by the rapid amplification of cDNA ends (RACE) method of Frohman (29) using the 5′-RACE System Kit (version 2.0, Life Technologies, Inc., Gaithersburg, MD). Based on the nucleotide sequence of the 3′ partial cDNA of putative salmon RFa, six antisense primers were synthesized as follows: sRF-R1, 5′-AAGCATGTTGTCATGGTAGCCA-3′; sRF-R2, 5′-AGAATCTCCAGTGTGCTGACCA-3′; sRF-R3, 5′-TCTTCCCAAACGCCCGATGG-3′; sRF-R4, 5′-ACGTACCAGAATGG-3′; sRF-R5, 5′-ACTTCTGTTGTCGACATTGT-3′; and sRF-R6, 5′-GAAAGCAGCAACAGAATT-3′. These six gene-specific primers and the abridged anchor primer provided in the kit were used for RT-PCR. PCR conditions were the same as those described above.

PCR-amplified cDNA products were electrophoresed on agarose gels and visualized by ethidium bromide staining. The cDNA was extracted and purified from agarose gels using a QIAEX II gel extraction kit (QIAGEN, Hilden, Germany), ligated into pT7 Blue T-Vector (Novagen, Madison, WI), and transformed into JM109 competent cells (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocols. Plasmids containing the insert were extracted from the bacterial cells using the QIAprep Spin Plasmid Kit (QIAGEN). The cDNA nucleotide sequence was determined by sequencing according to the dideoxy chain termination method with a DNA sequencer (model 377, PRISM, PE Applied Biosystems). DNASIS-Mac (Hitachi, Tokyo, Japan) was used for processing the sequence data, aligning the sequences, and calculating sequence identity.

Preparation of C-RFa antiserum and affinity column

C-RFa was synthesized using an automated solid phase peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan). A series of reactions was performed according to the manufacturer's protocols. The peptide was purified by HPLC on a reverse phase TSK gel ODS-120T column (0.46 × 25 cm, 5-μm particle size; TOSOH, Tokyo, Japan) with a linear gradient of 20–50% acetonitrile in 0.1% trifluoroacetic acid (TFA) for 60 min at a column temperature of 40 C and a flow rate of 1 ml/min. Absorbance was monitored at 220 nm. The amino acid sequence of the synthetic peptide was confirmed by sequence analysis using an automated gas phase protein sequencer (Shimadzu PSQ-1).

The synthetic C-RFa (2 mg) was conjugated with BSA (6 mg; Sigma, St. Louis, MO) in water by the carbodiimide method according to Goodfriend *et al.* (30) and was stored at −30 C after dialyzing in water. Each of the conjugated peptides (100 μg) was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected subdermally on the back of rabbits at intervals of 3 wk. Blood was collected 2 wk after the fifth injection, and the lyophilized serum was stored at −20 C.

The affinity column was prepared by coupling a partially purified antisynthetic C-RFa serum IgG fraction to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Isolation of salmon RFa

Pulverized frozen chum salmon hypothalami (115 g) were boiled in 1 liter distilled water for 10 min, and then 30 ml acetic acid were added. The material was homogenized with a blender homogenizer (5,000 rpm; Nihonseiki, Tokyo, Japan) for 30 min on ice. After centrifugation (10,000 × *g*, 40 min, 4 C), the supernatant was concentrated to 150 ml using a rotary evaporator under vacuum, poured into 500 ml prechilled ethanol, and allowed to stand for 15 min. After centrifugation to remove the precipitate, the supernatant was concentrated to 25 ml by a rotary evaporator, and 2 ml 1 N HCl were added. The resulting supernatant (15 ml each) was applied to three Sep-Pak C_{18} cartridges (Waters Corp., Milford, MA) equilibrated with 0.1% TFA. After washing the cartridges with 0.1% TFA, the adsorbed peptides were eluted with 60% acetonitrile in 0.1% TFA and then lyophilized. The fraction (15 mg) was dissolved in 50 mM $NaHCO_3$ containing 0.5 M NaCl (pH 8.3) and loaded into an immunoaffinity column coupled to antisynthetic C-RFa serum. The unadsorbed peptides (monitored by absorbance at 280 nm) were eluted with the loading buffer, and subsequently the adsorbed peptide was eluted with 0.1 N acetic acid. The adsorbed peptide was subjected directly to HPLC on an ODS-120T column using the same conditions as those described above. Salmon RFa was screened by immunoblotting with antisynthetic C-RFa serum diluted 1:5000, and the reactive fraction was visualized by staining of peroxide products after incubation with avidin:biotin/peroxidase reagent (Vector Laboratories, Inc., Burlingame, CA). The amino acid sequence at the N-terminal region of the immunoreactive peak was determined by an automated protein sequencer (Shimadzu PSQ-1).

Immunocytochemistry

Immature rainbow trout of both sexes (120 g BW) were used. Immunocytochemistry was conducted basically as described by Amano *et al.* (31) with a slight modification. Fish were anesthetized with 0.05% 2-phenoxyethanol, and brains with pituitary were fixed with Bouin's fluid without acetic acid at 4 C for 24 h. Subsequently, the tissue was dehydrated and embedded in paraplast (Monoject, Sherwood Medical, St. Louis, MO). Sagittal sections were cut at 5 μm on a microtome. Immunocytochemical staining of RFa neurons and fibers was carried out with antisynthetic C-RFa serum using a Histofine immunostaining kit (Nichirei, Tokyo, Japan). The adjacent section was also stained with antisalmon PRL and SL sera. Antisynthetic C-RFa serum was diluted

```
   1                                                    TGAATGAATAACCTGGCACGA   21
   1                                                       M  N  N  L  A  R     6

  22  GGAAAACTCTCCACCGACCCACCTCAGCAAGCTACCAACGTTTACGTGCCTTGCCGTCCA   81
   7   G  K  L  S  T  D  P  P  Q  Q  A  T  N  V  Y  V  P  C  R  P    26

  82  TCCACAATGACACCTGAGACTACAGCTGCGTGCCCAGTGATGGTGAGAGAGTGCGTCCTG  141
  27   S  T  M  T  P  E  T  T  A  A  C  P  V  M  V  R  E  C  V  L    46

 142  GGGTCCCGCTGGCTGATGGCTGCCCTCACAATTCTGTTGCTGCTTTCCACAACGGTCACC  201
  47   G  S  R  W  L  M  A  A  L  T  I  L  L  L  L  S  T  T  V  T    66

 202  TGCTTTCACAGTACCACCGTGGAACACAACTTTCACATCGTTCACAATGTCGACAACAGA  261
  67   C  F  H  S  T  T  V  E  H  N  F  H  I  V  H  N  V  D  N  R    86

 262  AGTCCAGAAATAGATCCATTCTGGTACGTGGGCCGTGGGGTAAGGCCCATCGGGCGTTTT  321
  87   S  P  E  I  D  P  F  W  Y  V  G  R  G  V  R  P  I  G  R  F   106

 322  GGGAAGAGGCAGAGCGGAGGAGGGGGCAGCGGAGGGCTCAGACACCCTGTGGCCATGGTC  381
 107   G  K  R  Q  S  G  G  G  G  S  G  G  L  R  H  P  V  A  M  V   126

 382  AGCACACTGGAGATTCTCCTCGACATCATCAGGAACCAGGAGAACATCGGGAAGACACTT  441
 127   S  T  L  E  I  L  L  D  I  I  R  N  Q  E  N  I  G  K  T  L   146

 442  AGCGGAGAGGACGCCGACTGGCTACCATGACAACATGCTTGGCCGCTCGCCCGCCCCCAC  501
 147   S  G  E  D  A  D  W  L  P  *                                  155
 502  CATCTGTGTCCGTGAACGTGTCTTTATTTAATCAAGTATTGTCTTTCTTTGTCTCTATCT  561
 562  CCTGTTTCTTTCTGTCATTCTTTCTTTCTTTTCAAGGTTTGCTCTCAAGCCTACAGTATG  621
 622  TCTTAGTCTAGGGACCTCTAAACTGGCCGACCAGTTGTAGTCTGTATGGTACATACTGAG  681
 682  AAATGCAATCTATATTGATTAATAAATGAAGATAGTAACAG                      722
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FIG. 1. Nucleotide sequence of salmon RFa cDNA without a poly(A) tail and the deduced amino acid sequence. The numbers of nucleotide and amino acid sequences are indicated on both sides of the line. The stop codon is marked with an *asterisk*. The polyadenylation signal site is *underlined*.

1:10,000, and anti-PRL and SL sera were diluted 1:5,000 with 0.1 M phosphate buffer (pH 7.4) containing 0.75% NaCl and 0.3% Triton X-100. To test the specificity of the immunoreaction, the control sections were incubated with anti-C-RFa serum that was preabsorbed overnight at 4 C with an excess amount of synthetic C-RFa (1 μg peptide in 1 ml antiserum).

Hypophysiotropic activity

Two milligrams of synthetic C-RFa were first dissolved in 200 μl distilled water and diluted to 0.5 μg/μl with 0.9% NaCl solution. The synthetic peptide was also dissolved in 1 ml HBSS (Sigma) with 25 mM HEPES, pH 7.0, and 1-pM to 100-nM solutions were prepared. All solutions were stored at −20 C until use.

As the basal level of PRL release is lower than that of GH in organ-cultured pituitary of the rainbow trout (25), the effect of C-RFa on PRL release was examined under isotonic condition.

Intraperitonal injection: in vivo effects

Immature rainbow trout of 100 g BW (five fish per group) were maintained in fresh water in indoor 50-liter tanks and fed a standard ration of 6P pellets (Nihon Nosan, Tokyo, Japan). Food was withheld 24 h before injection. Fish were anesthetized with 0.01% 2-phenoxyethanol and injected ip with either 50 or 500 ng synthetic C-RFa/g BW. Control fish received injection vehicle only (10 μl/g BW). Blood samples were collected from the caudal vessels at 1, 3, 6, 9, and 12 h after injection of the synthetic peptide. Plasma was separated immediately by centrifugation at 800 × *g* for 10 min and was stored at −80 C until assay.

Perifusion: in vitro effects

Six pituitaries from immature rainbow trout (300 g BW) were used. After dissection, pituitaries were washed twice with 1 ml ice-cold HBSS with 25 mM HEPES (pH 7.0). They were then placed separately on top of a steel screen (diameter, 1 cm) on each chamber for perifusion following methods described by Gazourian *et al.* (32). The Acusystem-S multiperfusion system (Endotronics, Inc., Minneapolis, MN) was used to deliver medium at 4 ml/h at 10 C to the 6 chambers at a constant volume (400 μl/6 min). Initially each chamber was perifused with buffer for 1 h (10 fractions) to obtain the basal release rate. Subsequently, the synthetic C-RFa at concentrations of 1 pM to 100 nM (100 μl) was perifused separately, and 15 fractions (400 μl each) were collected.

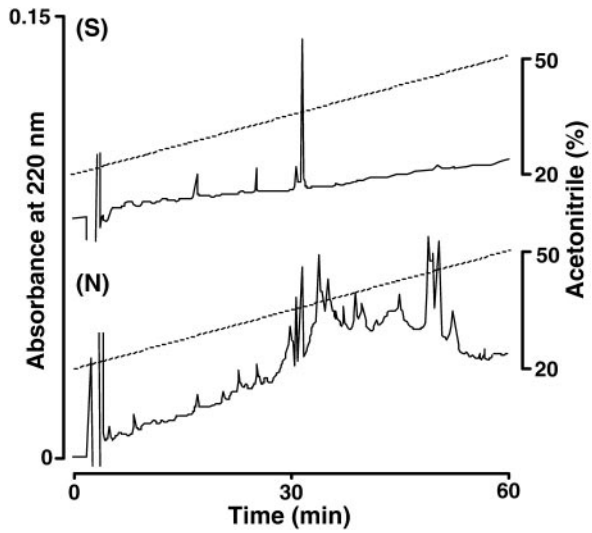

FIG. 2. Comparison of the elution positions of synthetic (S) and native (N) C-RFas applied onto a reverse phase ODS 120T column (0.46 × 25 cm, 10-μm particle size) at a column temperature of 40 C and a flow rate of 1 ml/min. The *dashed line* represents acetonitrile in 0.1% TFA.

RIAs

Concentrations of PRL, GH, and SL in plasma and perifusion fractions were measured by homologous RIAs according to the methods of Swanson (33) with some modification. Antibody-bound hormone complexes were separated from the free trace by the addition of 0.25% Pansorbin (Calbioche, Darmstadt, Germany). The lowest detectable level (ED_{80}) of PRL was 0.37 ng/ml, that of GH was 0.78 ng/ml, and that of SL was 0.64 ng/ml. All samples were measured in duplicate assays, with intraassay variation less than 4%.

Statistical analysis

All data are presented as the mean ± SE. Group comparisons were performed using one-way ANOVA, followed by Fisher's least signifi-

cant difference test. Differences at $P < 0.05$ or $P < 0.01$ were considered significant.

Results

cDNA cloning for salmon RFa

The cDNA fragment of 398 bp (nucleotides 324–722) was amplified in the first PCR using the degenerate forward sRF-1 and *Not*I adapter primers (Fig. 1). The second PCR using 5′-RACE reverse primers and 5′-RACE abridged anchor primer yielded a product of 376 bp that spanned from the beginning of the 5′-end and overlapped with the known sequence of the 3′-region. Excluding the poly(A) tail, the putative salmon RFa cDNA consisted of 722 nucleotides. This was composed of 3 bp in the 5′-untranslated region, a 465-bp open reading frame, and a 254-bp 3′ untranslated region including the hexanucleotide AATAAA (nucleotides 702–707), the typical polyadenylation signal. The open reading frame of salmon RFa cDNA encoded 155 amino acids. The typical proteolytic cleavage sequence, lysine and arginine, was located at positions 86 and 108–109; the C-terminal amidation motif (Gly-Lys-Arg) was also identified at positions 107–109, suggesting that the putative salmon RFa is identical with C-RFa.

Isolation of salmon RFa

Figure 2 shows reverse-phase HPLC pattern of the adsorbed peptide from immunoaffinity chromatography compared with synthetic C-RFa. Cross-reaction with antisynthetic C-RFa serum was observed with the fraction that eluted at 32 min (36% acetonitrile concentration). Amino acid sequencing of this peptide corresponded to 15 amino acids, positions 87–101. The elution position of the purified peptide was identical with that of a synthetic C-RFa. The total yield of salmon RFa was 4 pmol from 115 g of the hypothalami based on the yield of amino acid sequence analysis.

The amino acid sequence of salmon prepro-RFa was compared with those of bovine PrRP (27) and Japanese crucian carp and tilapia, *Oreochromis mossambicus*, RFas (Refs. 28 and 34 and Fig. 3). The putative salmon RFa was identical with that of C-RFa, and 13 amino acids were identical with those of bovine PrRP 20.

Immunocytochemistry

The distribution of C-RFa-immunoreactive cell bodies and fibers is summarized in Fig. 4. The C-RFa-immunoreactive cell bodies were found in the posterior hypothalamus, and immunoreactive fibers were abundant from the hypothalamus to the ventral telencephalon (Fig. 5A). A small number of immunoreactive fibers were projected to the rostral part of the pituitary gland and terminated close to PRL cells (Fig. 5, B and C) and SL cells (Fig. 5, C and D). No immunoreactive cell body and fiber were observed with the antibody preabsorbed with excess amount of synthetic C-RFa (data not shown).

```
                1                                                          60
Salmon RFa    : MNNLARGKLS TDPPQQATNV YVPCRPSTMT PETTAACPVM VRECVLGSRW LMAALTILLL
Carassius RFa:                                   MLPT*ITQP *TK*LI***L GTI*FLL**I
Tilapia RFa   :                                  MLPVRAAD- *RH***T*** *P***AL***
Bovine PrRP   :                                                MKA VG*W*LC***

               61                                  RFa                    120
Salmon RFa    : LSTTVTCFHS TTVEHNFHIV HNVDNRSPEI DPFWYVGRGV RPIGRFGKRQ SGGGGSGGLR
Carassius RFa: **A*ESNA*G *****DL*** ********** ********** *********Q ****----*-
Tilapia RFa   : **SSFSRA** *****D**** ********** ********** *******H *SLEALDSDG
Bovine PrRP   : *------GLA LQGAASRAHQ *SMEI*T*D* N*A**A***I **V****R*R AAP*--D*P*
                                        PrRP
              121                                 155
Salmon RFa    : HPVAMVSTLE ILLDIIRNQE NIGKTLSGED ADWLP
Carassius RFa: Q**--*KS** ***NTL**K* SLRSA*AQ*E S****
Tilapia RFa   : M**--*RT** L**SSL**K* *L**V*D*** *
Bovine PrRP   : PGPRR*PACF R*EGGAEPSR ALPGR*TAQL VQE
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FIG. 3. Sequence comparison of salmon prepro-RFa with Japanese crucian carp (28) and tilapia (34) RFas and bovine prepro-PrRP (2). The *asterisk* represents identical residues as salmon prepro-RFa. The *dashed line* indicates a gap.

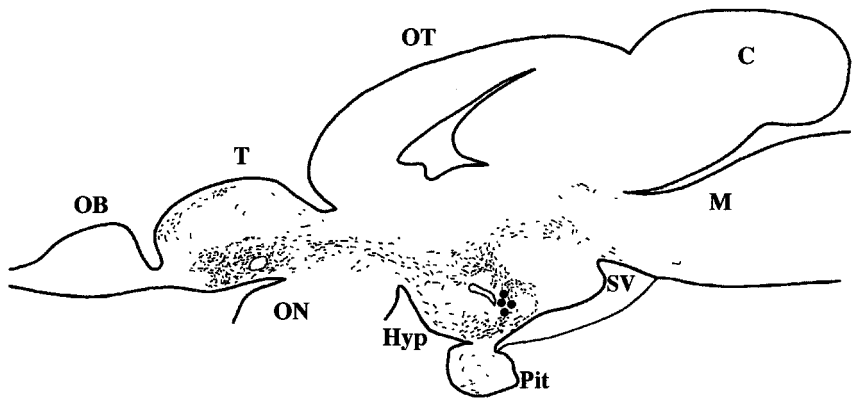

FIG. 4. Schematic distribution of C-RFa-immunoreactive cell bodies (*dots*) and fibers (*line*) in a sagittal section. C, Cerebellum; Hyp, hypothalamus; M, medulla oblongata; OB, olfactory bulb; Olf, olfactory nerve; ON, optic nerve; OT, optic tectum; PIT, pituitary; SV, saccus vasculosus; T, telencephalon.

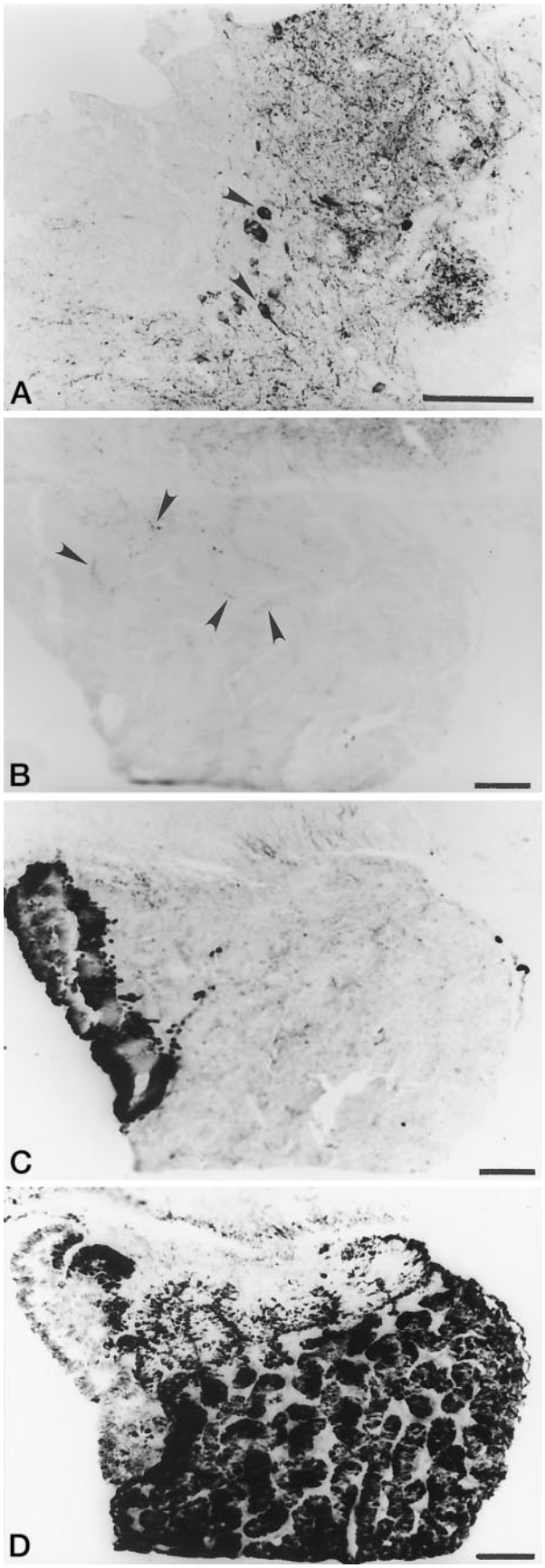

FIG. 5. A, C-RFa-immunoreactive cell bodies (*arrowhead*) and fibers (*arrowhead*) in the posterior part of the hypothalamus. B, C-RFa-immunoreactive fibers in the pituitary. C, PRL-immunoreactive cells in the rostral pars distalis of the pituitary. D, SL-immunoreactive cells in the pars intermedia of the pituitary. *Bar*, 100 μm.

Hypophysiotropic activity

Intraperitoneal injection of immature rainbow trout with synthetic C-RFa at doses of 50 and 500 ng/g resulted in a significant ($P < 0.01$) elevation of plasma PRL levels after 3 h compared with the saline-injected control value (Fig. 6A). PRL levels declined gradually to reach normal levels after 12 h. The PRL levels in fish injected with 500 ng/g were higher than those in fish injected with 50 ng/g, although the difference was not statistically significant. A significant ($P < 0.01$) increase in plasma SL levels was observed after 9 h at both 50 and 500 ng/g (Fig. 6B). In contrast, plasma GH levels declined significantly ($P < 0.01$) after 1 h at 500 ng/g, whereas no change was observed at 50 ng/g (Fig. 6C).

PRL releases increased significantly when the pituitaries were perifused with synthetic C-RFa at doses of 10 pM, 100 pM, 10 nM, and 100 nM (Fig. 7A). The highest PRL release was observed at 100 pM ($P < 0.01$). SL release was also stimulated at doses of 10 nM ($P < 0.01$) and 100 nM ($P < 0.01$; Fig. 7B). However, GH release was not affected (Fig. 7C).

Discussion

In the present study we identified RFa by cDNA cloning and peptide isolation in chum salmon, compared the sequences to those of newly identified RFas in Japanese crucian carp (28) and tilapia (34), and determined that salmon RFa is identical with C-RFa (8). We further demonstrated by immunocytochemistry that the distribution of C-RFa neuronal terminals was located near PRL and SL cells in the pituitary. The hypophysiotropic activity of C-RFa determined by ip injection and pituitary perifusion in rainbow trout clearly demonstrated that of the various pituitary hormones tested, PRL release occurred both *in vivo* and *in vitro*. Together with the results obtained from tilapia (34), we propose that these data are the first to demonstrate that a homolog of mammalian PrRP (fish RFa) is a major hypothalamic peptide of PRL release in teleost fish.

Identification of salmon RFa

In this study salmon prepro-RFa consisted of 155 amino acid residues, which is 38 residues longer than preprohormones of Japanese crucian carp (28) and tilapia (34). Salmon prepro-RFa-(86–105) was identical with the sequence of C-RFa, followed by C-terminal amidation motif at positions 107–109 (Gly-Lys-Arg). Additional cleavage sites were predicted between positions 67 (Cys) and 68 (Phe) or positions 69 (His) and 70 (Ser), and between 85 (Arg) and 86 (Ser) according to the method of Nielsen *et al.* (35). This analysis suggested the occurrence of another putative RFa of 37 or 36 residues, which is highly conserved among teleosts, in addition to the C-RFa consisting of 20 amino acids. Because similar cleavage sites were also predicted for crucian carp and tilapia RFa precursors, we carefully analyzed the extract

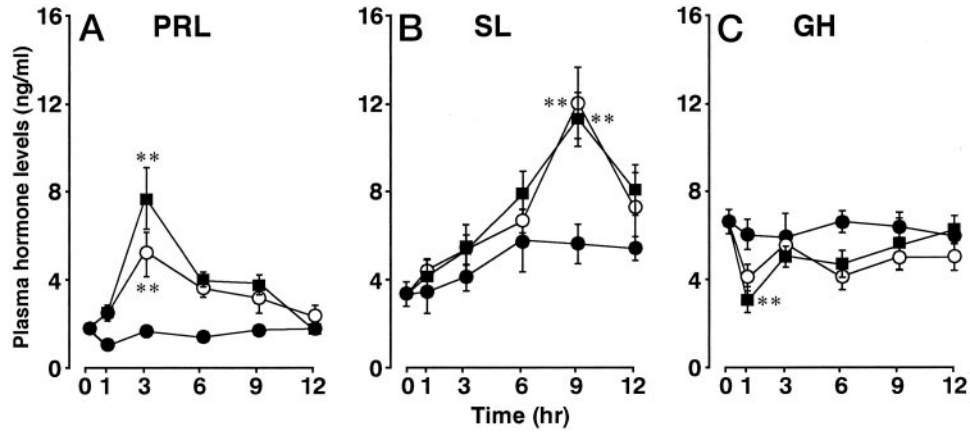

FIG. 6. Changes in plasma levels of PRL (A), SL (B), and GH (C) in rainbow trout (100 g; n = 5) after ip injection of a synthetic C-RFa at a dose of 50 or 500 ng/g BW. ○, ■, and ●, Groups that received 50, 500, and 0 ng/g BW synthetic C-RFa, respectively. Each *point* represents the mean ± SEM. **, Significant difference between C-RFa-injected fish and control ($P < 0.01$).

of salmon hypothalami. However, only the 20-amino acid RFa was isolated, as shown in Fig. 2. These results suggested that, unlike mammalian PrRP, a longer molecular form may not be produced in these teleosts.

Immunocytochemical analysis of RFa in the rainbow trout

In the rat, PrRP cells are located in the medulla oblongata and the hypothalamus, and the fibers containing PrRP are widely distributed in the brain (7, 36–38). A small number of fibers were also observed in the neuronal lobe of the pituitary, but no fiber was observed in the external layer of the median eminence, which is the release site of the classical hypophysiotropic hormone (36).

In the rainbow trout, C-RFa somata were located in the basal hypothalamus, and the fibers were projected widely from the hypothalamus to the ventral telencephalon, as reported in the goldfish by Satake *et al.* (28) and Wang *et al.* (39). Therefore, C-RFa may play a role as a neurotransmitter or a neuromodulator in teleosts as well as mammals (36–38). In the rat it has been demonstrated that PrRP neurons make synapse-like contact with neurons of oxytocin (36), CRH (5), and somatostatin (7). Further experiments showed that intracerebroventricular administration of PrRP stimulated the release of oxytocin (3) and ACTH (5) while inhibiting GH (7). However, a small number of PrRP nerve fibers were detected in the posterior pituitary, but not in the external layer of the median eminence or in the anterior pituitary (36). To interpret the hypophysiotropic effect of this peptide on PRL cells, Maruyama *et al.* (36) speculated that PrRP might be transported to its receptor site in the anterior pituitary via the posterior pituitary through a short hypophyseal portal system.

The teleost pituitary gland is divided into three parts, rostral pars distalis, proximal pars distalis, and pars intermedia, where PRL-, GH-, and SL-producing cells are localized. In the present study a few immunoreactive axon terminals were detected in the rostral pars distalis and the pars intermedia close to PRL- and SL-producing cells.

Hypophysiotropic effects of C-RFa

On the basis of the localization of C-RFa axon terminal, we compared the hypophysiotropic effects on the release of three evolutionarily related hormones, PRL, GH, and SL, in the rainbow trout.

Effects on PRL release. The synthetic C-RFa induced PRL release both *in vivo* and *in vitro* in the rainbow trout. A significant elevation of plasma PRL levels was observed 3 h after ip injection. This elevation was greater at a larger dose. Furthermore, PRL release was stimulated immediately after perifusion of the trout pituitary with this peptide. Maximum PRL release was observed at 100 pM. In the tilapia, C-RFa stimulated the release of two PRLs, PRL177 and PRL188, *in vitro* from the rostral pars distalis of tilapia pituitary at doses of 10 or 100 nM under hyperosmotic conditions (360 mosmol/kg), in which PRL release is suppressed (34).

There is some evidence for the gender-biased effect of PrRPs in mammals. In the rat pituitary cell culture, PrRPs at a dose of 0.1 or 1 μM stimulated PRL secretion in females, but not in males (40). Matsumoto *et al.* (3) also reported that the stimulatory effect of PrRP on PRL release was affected considerably by the estrous cycle and the sex of the rat after iv injection of PrRP30. Seale *et al.* (34) reported that the effect of C-RFa on PRL release in the tilapia was different between males and females, with the effect in the male being more pronounced than that in females. However, the injection of C-RFa induced an elevation of plasma PRL levels only in females (34). In the present study the difference in response to C-RFa between male and female trout could not be ascertained, because we used immature fish.

Effects on SL release. C-RFa was also effective in stimulating SL release from the rainbow trout pituitary. SL is a multifunctional pituitary hormone presumably involved in reproduction (19, 20), calcium regulation (21), stress response (22), adaptation to the environment (23, 24), and acid/base balance (25, 26). However, no hypothalamic factor is known to regulate SL release. Kakizawa *et al.* (25) reported that SL release was inhibited by dopamine and epinephrine in the organ-cultured pituitary of rainbow trout, and some hypothalamic peptides, such as serotonin and CRF, stimulated SL release in the dopamine-inhibited pituitary. The present study demonstrated the stimulation of SL release by C-RFa *in vitro*. The elevation of plasma SL levels was observed 9 h after injection. The increase was observed later than the increase in PRL levels. It is possible that the effect of C-RFa on

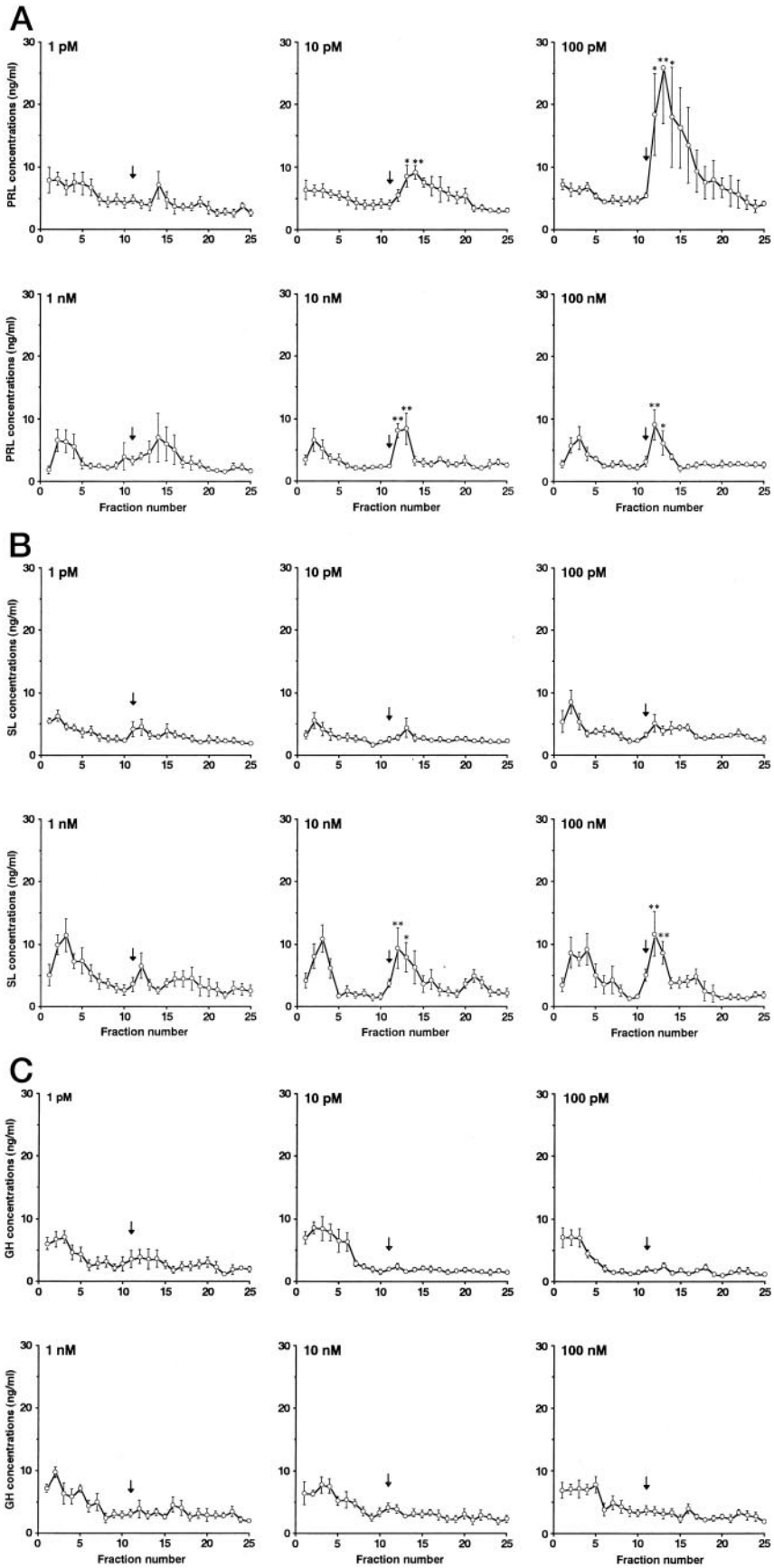

FIG. 7. Effects of the synthetic C-RFa on release of PRL (A), SL (B), and GH (C). Initially, each pituitary (n = 6) was perifused with HBSS in 25 mM HEPES (pH 7.0) at 4 ml/h at 10 C. Ten fractions were collected to obtain the basal release rate of the hormones, and then synthetic C-RFa at concentrations of 1 pM to 100 nM was perifused separately. The *arrow* represents the application of the C-RFa. Each *point* represents the mean ± SEM. Significant differences from the control (fraction 10) are indicated (*, $P < 0.05$; **, $P < 0.01$).

SL release is mediated indirectly or by some other mechanism. Indeed, after injection of C-RFa, the elevation of plasma SL levels did not show a dose dependency. The stimulatory effect of C-RFa on SL release was less pronounced than that on PRL release *in vitro*. Given all of these possibilities, a more detailed investigation of the effect of C-RFa on SL release is necessary.

Effects on GH release. Plasma GH levels were decreased 1 h after the injection of C-RFa at a dose of 500 ng/g. However, no change in GH release was observed *in vitro*. In rats, PrRP neurons are connected to somatostatin neurons (7). In addition, PrRP inhibited GH release from the pituitary (7). Taken together, these results show that C-RFa inhibits GH release, as it does in mammals.

In conclusion, we have demonstrated that chum salmon RFa is identical with Japanese crucian carp and tilapia RFas by cDNA cloning and peptide isolation from the hypothalamus. Immunohistochemical localization of RFa cells and fibers suggested a role for the peptide as a neurotransmitter or a neuromodulator and hypophysiotropic factor for PRL and SL. Administration of C-RFa stimulated PRL and, to a lesser degree, SL release from the pituitary in the rainbow trout. C-RFa appeared to inhibit GH release. These results provide evidence that C-RFa is a major hypothalamic peptide involved in PRL release in rainbow trout.

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Address all correspondence and requests for reprints to: Shunsuke Moriyama, Ph.D., Laboratory of Molecular Endocrinology, Kitasato University School of Fisheries Sciences, Sanriku, Iwate 022-0101, Japan. E-mail: morisuke@kitasato-u.ac.jp.

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