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Isolation and Structural Characterization of Two Novel Peptides Derived from Proopiomelanocortin in the Pituitary of the Rainbow Trout

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The trout possesses two POMC genes as a result of duplication of its genome some 25-100 million years ago. One of the POMC molecules exhibits a unique C-terminal extension of 25 amino acid residues which is not found in any other POMC characterized so far. In order to isolate possible novel peptides derived from trout POMC-A, we have raised antibodies against two synthetic epitopes derived from the C-terminal region of the precursor. Two native decapeptides were isolated in pure form from an extract of trout pituitary. The primary structures of these peptides were established as Glu-Gln-Trp-Gly-Arg-Glu-Glu-Gly-Glu-Glu and Ala-Leu-Gly-Glu-Arg-Lys-Tyr-His-Phe-Gln-NH_2. The structure of the trout POMC-A cDNA reveals that both peptides are flanked by pairs of basic amino acids or a glycine residue, indicating that they can actually be generated during post-translational processing of POMC-A.

Proopiomelanocortin (POMC) is the common precursor protein for a number of peptide hormones and neuropeptides including corticotropin (ACTH), alpha-melanotropin (α-MSH) and β-endorphin [1,2]. Molecular cloning of the POMC cDNAs from various representative species has revealed that the structure of POMC has been highly conserved during evolution [2-11]. Some vertebrate species which underwent chromosome duplication, such as the toad *Xenopus laevis,* the salmon and the trout, possess two POMC genes [9-11]. In the trout, one of the POMC proteins exhibits a unique C-terminal extension of 25 amino acids [11] which is not found in any other POMC characterized so far (Fig. 1). This C-terminal extension contains three pairs of basic residues which represent potential cleavage sites for prohormone convertases [12]. This suggests that post-translational processing of trout POMC-A may generate several novel peptide hormones and/or neuropeptides. In addition, the C-terminal amino acid of POMC-A is a glycine residue so that some of these potential peptides may be α-amidated [13]. Since many biologically active peptides are α-amidated, we hypothesized that the C-terminal extension of trout POMC-A may generate novel regulatory peptides [14].

In order to characterize these potential novel peptides, we have raised antibodies against two synthetic epitopes derived from the C-terminal extension of trout POMC-A. The present report describes the isolation and structural characterization of two as yet unknown decapeptides from an extract of trout pituitary.

MATERIALS AND METHODS

Reagents. The Rink amide 4-methylbenzhydrylamine resin used for C-terminal α-amidated peptide synthesis was purchased from Biochem (Meudon, France). The Fmoc-Glu-HMP resin used for C-terminal-COOH peptide synthesis was obtained from Applied Biosystems (St. Quentin en Yvelines, France). Bovine thyroglobulin, glutaraldehyde and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co (St. Louis, MO). $Na^{125}I$ was from Amersham (Les Ulis, France). Bovine serum albumin (BSA; fraction V) was purchased from Boehringer (Paris, France). Acetonitrile was from

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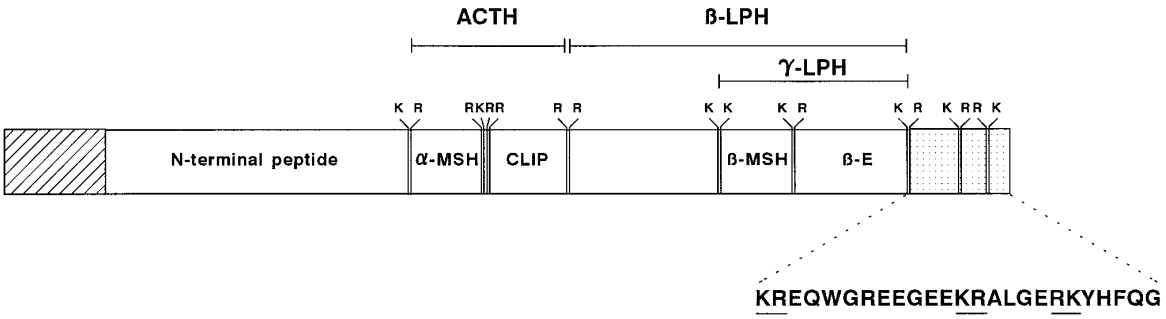

FIG. 1. Schematic representation of the structure of trout pre-POMC-A. Vertical bars indicate pairs of basic amino acids. The hatched zone represents the signal peptide sequence. The dotted zone represents the C-terminal extension of POMC-A. The primary structure of this 25 amino acid peptide is shown, and the three pairs of basic amino acids are underlined.

Carlo Erba (Milan, Italy). Fmoc-protected amino acids were from Applied Biosystems.

Peptide synthesis and production of antibodies. The following four peptides were synthesized using the solid phase methodology on a 433A Applied Biosystems model synthesizer: EQWGREEGEE and its N-terminally tyrosylated analog YEQWGREEGEE; YHFQ-NH_2 and its N-terminally extended form ALGERKYHFQ-NH_2 [15]. Each peptide was purified to apparent homogeneity on a Vydac C_{18} column (>99% pure) and characterized by mass spectrometry. The peptides EQWGREEGEE and YHFQ-NH_2 were conjugated to bovine thyroglobulin using glutaraldehyde as a coupling agent. After a 2-hour incubation in phosphate buffer (pH 7.5), the conjugate was purified by dialysis. The coupling efficiency was 79% for EQWGREEGEE and 85% for YHFQ-NH_2. Antibodies against each conjugate were raised in New Zealand rabbits (CEGAV, St. Mars d'Egrenne, France). The animals were initially injected with an equivalent of 200 μg of peptide emulsified with Freund's complete adjuvant. Subsequent injections were made at monthly intervals using a mixture of incomplete adjuvant with 200 μg of peptide. The antisera against EQWGREEGEE and YHFQ-NH_2 selected for radioimmunoassay (RIA) gave approximately 25% specific binding of the tracers at final dilutions of 1:85,000 and 1:12,000, respectively. The concentrations of EQWGREEGEE- and ALGERKYHFQ-NH_2-like immunoreactivity in HPLC fractions from trout pituitary extracts were measured by using double-antibody RIA methods as previously described [16]. The IC_{50} of the RIAs were 35.8 fmole and 1930 fmole/tube, respectively.

Tissue extraction. Thirty adult rainbow trout, *Oncorhynchus mykiss*, of both sexes were obtained from a fish farm (Montville, France). The animals were killed by decapitation and pituitaries were removed and immediately frozen on dry ice. The tissue was immersed in 2 ml boiling 2 M acetic acid and maintained in a boiling water for 10 min to ensure inactivation of proteolytic enzymes. The tissue was homogenized at 4°C using a glass Potter homogenizer and sonicated. After centrifugation (13,000 × g for 30 min at 4°C), peptide material contained in the supernatant was concentrated on a Sep-Pak C_{18} cartridge as previously described [17].

Purification of trout POMC-derived peptides. The pituitary extract was chromatographed on a Vydac 218TP1010 C_{18} column (250 × 10 mm, Separations Group, Hesperia, CA) equilibrated with acetonitrile/water/TFA (10.0:89.9:0.1; v/v/v) at a flow rate of 2 ml/min. The concentration of acetonitrile was held at 10% for 10 min and raised to 50% over 60 min using a linear gradient. Absorbance was measured at 215 and 280 nm and fractions (2 ml) were collected. The concentrations of EQWGREEGEE- and ALGERKYHFQ-NH_2-like immunoreactivity in the fractions were measured by RIA at appropriate dilutions.

The fractions containing the highest concentrations of EQWGREEGEE-like immunoreactivity were pooled (fraction F_1 in Fig. 2) and rechromatographed on a Lichrosorb C_{18} column (250 × 4 mm, Merck, Darmstadt, Germany) equilibrated with acetonitrile/water/TFA (10.0:89.9:0.1; v/v/v) at a flow rate of 1 ml/min. The concentration of acetonitrile was held at 10% for 10 min and raised to 34% over 30 min using a linear gradient. Individual peaks were collected by hand without using a fraction collector (data not shown). The peak exhibiting EQWGREEGEE-like immunoreactivity was rechromatographed on the same column with the same equilibration conditions and flow rate. The concentration of acetonitrile was held at 10% for 10 min, raised to 16% over 6 min and then to 30% over 20 min. The peak which exhibited EQWGREEGEE-like immunoreactivity (peak I in Fig. 3A) was sequenced.

The fraction containing ALGERKYHFQ-NH_2-like immunoreactivity (fraction F_2 in Fig. 2) was rechromatographed using the conditions described for fraction F_1. Individual peaks were collected by hand and the peak exhibiting ALGERKYHFQ-NH_2-like immunoreactivity was rechromatographed on the same column with the same equilibration conditions. The concentration of acetonitrile in the eluting solvent was held at 10% for 10 min, raised to 20% over 10 min and then to 34% over 20 min. The peak which exhibited ALGERKYHFQ-NH_2-like immunoreactivity (peak II in Fig. 3B) was sequenced.

Structural analysis. The primary structure of the two immunoreactive peptides (approximately 100 pmole of peak I and 250 pmole of peak II) was determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin amino acids under gradient elution conditions. Mass spectrometry analysis was performed on a Voyage RP MALDI-TOF instrument (Perspective Biosystems Inc., Framingham, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25 kV. Sample preparation was done as follows: 1 μl sample-solution (approx. 50 pmoles) was mixed with 10 μl matrix-solution (α-cyano-4-hydroxycinnamic acid dissolved in a 5:4:1 mixture of acetonitrile/water/3% TFA) and 1 μl was deposited on the same sample plate and allowed to dry.

RESULTS

Peptide purification. The elution profile on a semi-preparative Vydac C_{18} column of the extract of trout pituitaries is shown in Fig. 2. Fractions 26 and 27 denoted by the filled bar, associated with the EQWGREEGEE-like immunoreactivity, were pooled (fraction F_1) and selected for further purification. After two steps of

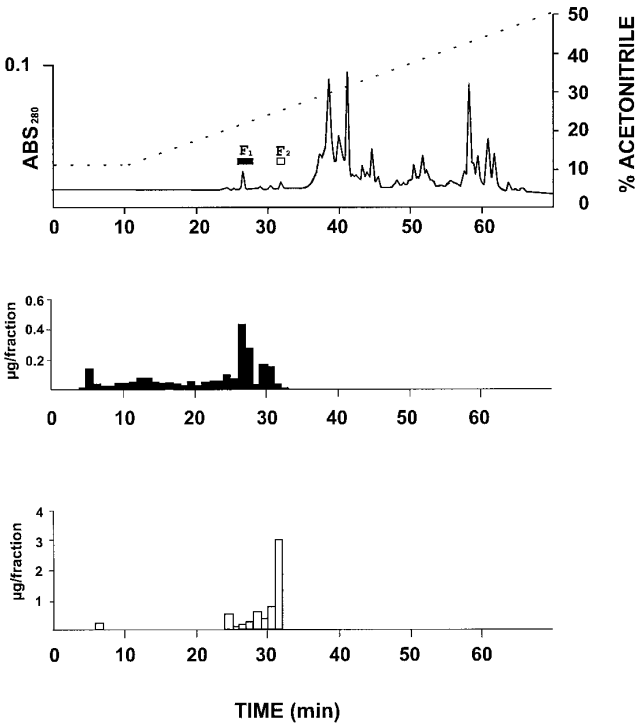

FIG. 2. Reversed phase HPLC on a Vydac C_{18} semi-preparative column of an extract of trout pituitaries after partial purification on a Sep-Pak cartridge. The histograms show the concentrations of EQWGREEGEE-like immunoreactivity (black bars, F_1) and ALGERKYHFQ-NH_2-like immunoreactivity (open bars, F_2). Fractions F_1 and F_2 were selected for further purification. The dashed line shows the concentration of acetonitrile in the eluting solvent.

purification on an analytical LiChrosorb C_{18} column, the immunoreactivity was associated with the peak denoted by the asterisk (peak I; Fig. 3A) and the final yield of pure material was 250 pmoles. Fraction 32 denoted by the open bar (fraction F_2), characterized by its ALGERKYHFQ-NH_2-like immunoreactivity, was selected for further purification. After two steps of purification the immunoreactivity was associated with a single peak denoted by the asterisk (peak II; Fig. 3B) and the final yield of pure material was 800 pmoles.

Structural characterization. The complete primary structure of the two peptides was determined by Edman degradation (Table 1). For each peptide, unambiguous assignment of amino acid phenylthiohydantoin derivatives was possible for 10 cycles of operation of the sequencer and no traces of additional residues were detected during cycle 11. The amino acid sequence of peptide II was confirmed by the results of mass spectometry. The observed molecular mass of peptide II was 1246.6 ± 1.0 compared with a calculated mass of 1247.4 for the COOH-terminal α-amidated form of the proposed sequence.

DISCUSSION

Molecular cloning of trout POMC-A has revealed the existence of an unusual C-terminal extension that has not been described in any other POMC molecules including trout POMC-B [11]. The presence of three pairs of basic residues within the sequence of this C-terminal tail suggested that it may be processed to as yet unknown peptides [11]. In order to identify some of these peptides, we have raised antibodies against two synthetic fragments derived from the C-terminal extension of trout POMC-A. Using these antibodies, we have been able to isolate in pure form two peptides by combining HPLC purification and RIA detection. Determination of

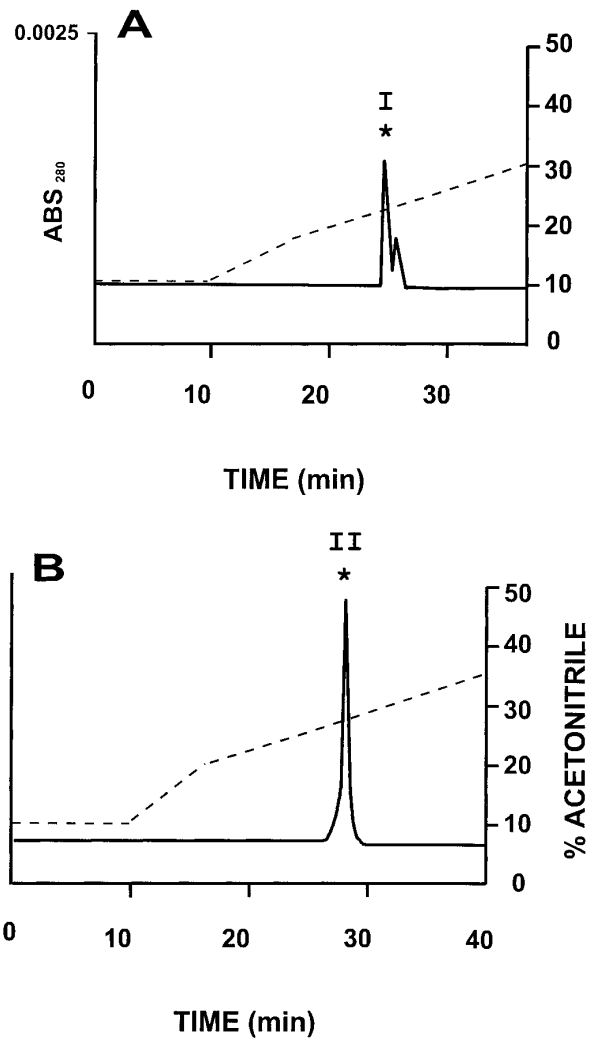

FIG. 3. Reversed-phase HPLC on an analytical Lichrosorb C_{18} column of fraction F_1 (A) and fraction F_2 (B) eluted from the semi-preparative column. The asterisks denote the peaks containing EQWGREEGEE-like immunoreactivity (A; peak I) and ALGERKYHFQ-NH_2-like immunoreactivity (B; peak II). The sequence of these two peptides were determined by automated Edman degradation.

TABLE 1

Determination of the Primary Structures of Peptides I and II from Trout Pituitary by Automated Edman Degradation

	Peptide I		Peptide II	
Cycle No.	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Glu	103	Ala	241
2	Gln	78	Leu	170
3	Trp	29	Gly	177
4	Gly	102	Glu	198
5	Arg	41	Arg	126
6	Glu	33	Lys	87
7	Glu	39	Tyr	143
8	Gly	45	His	91
9	Glu	25	Phe	127
10	Glu	29	Gln	61

the primary structure of these native peptides revealed that one of them (Glu-Gln-Trp-Gly-Arg-Glu-Glu-Gly-Glu-Glu) corresponds to the N-terminal sequence of the C-terminal extension. Within the POMC-A molecule, this peptide is flanked by two Lys-Arg dibasic sites indicating that it is actually generated *in vivo*. This decapeptide possesses five Glu residues and is thus a very acidic peptide. It has been previously shown that post-translational processing of gastrin generates a C-terminal peptide which also contains a core of five Glu residues in its sequence [18]. Interestingly, it has recently been reported that this latter anionic peptide acts cooperatively with gastrin to stimulate proliferation of tumoral pancreatic cells [19].

The sequence of the other peptide derived from the C-terminal extension of trout POMC-A was established as Ala-Leu-Gly-Glu-Arg-Lys-Tyr-His-Phe-Gln, and the determination of its mass by MALDI-TOF revealed that this peptide was C-terminally α-amidated. Consistent with this observation, trout POMC-A possesses a Gly residue at its C-terminus (Fig. 1) indicating that peptides derived from the C-terminal extension can be α-amidated.

In situ hybridization studies have shown that both POMC-A and POMC-B mRNA are present in corticotrope and melanotrope cells of the trout pituitary [11]. In contrast, the POMC genes are differentially expressed in the hypothalamus [11] suggesting that, in the trout brain, the neurons expressing the POMC-A and POMC-B genes may be involved in different functions. Antibodies directed against the C-terminal extension of POMC-A will be valuable tools to determine the distribution of the axon processes and nerve terminals containing POMC-A-derived peptides and thus the possible sites of action of these neuropeptides.

The characterization of two novel decapeptides derived from trout POMC-A raises the question of their potential biological activity. In particular, the decapeptide ALGERKYHFQ-NH_2 exhibits several characteristics of biologically active peptides: (*i*) it is C-terminally α-amidated and (*ii*) it is located at the C-terminal extremity of the precursor like many other regulatory peptides such as β-endorphin, dynorphin B, somatostatin, melanin-concentrating hormone, α-mating factor, etc. . . . Since POMC-A is expressed in both pituitary cells and hypothalamic neurons, the novel decapeptides may play either hormonal or neuronal functions of their own, in addition to the corticotropic, melanotropic and opioid activities of the other POMC-derived peptides. Alternatively, these novel peptides may modulate the biological activity of ACTH, α-MSH or β-endorphin. Consistent with this latter hypothesis, it has been previously shown that one of the pro-TRH-derived peptides termed PS_4 potentiates the effect of TRH on pituitary thyrotrope cells [20].

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