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Characterization of melanotropin-release-inhibiting factor (melanostatin) from frog brain: Homology with human neuropeptide Y

(peptide isolation/peptide mapping/pituitary melanotrophs/control of pigmentation/evolution)

NICOLAS CHARTREL*, J. MICHAEL CONLON†, JEAN-MICHEL DANGER*, ALAIN FOURNIER‡, MARIE-CHRISTINE TONON*, AND HUBERT VAUDRY*§

*European Institute for Peptide Research, Laboratory of Molecular Endocrinology, Centre National de la Recherche Scientifique URA 650, Unité affiliée à l'Institut National de la Santé et de la Recherche Médicale, University of Rouen, 76134 Mont-Saint-Aignan, France; †Regulatory Peptide Center, Department of Biomedical Sciences, Creighton University Medical School, Omaha, NE 68178; and ‡Université du Québec, Institut National de la Recherche Scientifique-Santé, Pointe-Claire, PQ, Canada H9R1G6

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ABSTRACT **A polypeptide was purified from frog brain extracts on the basis of its ability to inhibit α-melanotropin release from perifused frog neurointermediate lobes. Based on Edman degradation, amino acid analysis, and peptide mapping, the primary structure of this frog melanotropin-release-inhibiting factor (melanostatin) was determined to be H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Lys-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2. Frog melanostatin belongs to the pancreatic polypeptide/neuropeptide Y/peptide YY family, and the structure of this peptide differs from that of human neuropeptide Y by only one amino acid substitution in position 19. A synthetic replicate of frog melanostatin is coeluted with the native peptide on HPLC and is highly potent in inhibiting α-melanotropin secretion *in vitro* (IC_{50} = 60 nM).**

Both the anterior lobe and the intermediate lobe of the pituitary are regulated by hypophysiotropic factors originating from the hypothalamus (1, 2). Five families of hypothalamic neuropeptides involved in the control of anterior pituitary hormone secretion have now been characterized, namely, thyrotropin-releasing hormone (3, 4), gonadotropin-releasing hormone (5), somatostatin (6), corticotropin-releasing hormone (7), and growth hormone-releasing hormone (8, 9). Early experimental observations have demonstrated that the intermediate lobe of the pituitary is under hypothalamic inhibitory control (10, 11), and dopamine has been shown to play a pivotal role in the negative regulation of pituitary melanotrophs (12, 13). Although the pars intermedia of various species is richly innervated by oxytocin-containing (14) or mesotocin-containing (15, 16) fibers, neither oxytocin (17) nor mesotocin (16) can account for the inhibitory control of pituitary melanotrophs. In contrast, porcine neuropeptide Y (NPY) appears to be a potent inhibitor of α-melanotropin release in frog (18) and toad (19). Several other regulatory peptides characterized from hypothalamic extracts have been proposed as potential regulators of melanotropin release (20–22). However, many studies failed to demonstrate that these peptides meet the criteria expected of a physiological melanotropin-release-inhibiting factor (melanostatin) (23–25). We report here the purification, sequence analysis, and total synthesis of a 36-residue peptide that is highly potent in inhibiting the secretion of α-melanotropin from frog neurointermediate lobe *in vitro*.

MATERIALS AND METHODS

Purification Procedure. Adult frogs (*Rana ridibunda*) were obtained from a commercial source (Couétard, St-Hilaire de Riez, France). The brains from 1200 animals (94.5 g, wet weight) were collected on dry ice and kept frozen. The tissue was boiled for 15 min in 0.5 M acetic acid and homogenized in a Waring blendor. After centrifugation (10,000 × *g* for 30 min at 4°C), the supernatant was pumped at a flow rate of 2 ml/min through ten Sep-Pak C_{18} cartridges (Waters Associates) connected in series. Bound material was eluted with 70% (vol/vol) acetonitrile in water and lyophilized. The dry extract was dissolved in 10 ml of 1% (vol/vol) trifluoroacetic acid and chromatographed on a 2.5 × 100-cm column of Sephacryl S-100 (Pharmacia-LKB) equilibrated with 1 M acetic acid at a flow rate of 2 ml/min. Fractions (10 ml) were collected and absorbance was measured at 280 nm (Fig. 1*A*). The fractions exhibiting melanotropin-release-inhibiting activity were pooled and 50% of the total material was analyzed by HPLC.

The active fraction was pumped at a flow rate of 2 ml/min onto a 1 × 25-cm Vydac 218 TP510 C_{18} column (The Separations Group) equilibrated with 0.1% trifluoroacetic acid and eluted at a flow rate of 2 ml/min, using the gradient indicated in Fig. 1*B*. Absorbance was measured at 214 and 280 nm, and individual peaks were collected by hand. The peak containing melanotropin-release-inhibiting activity was rechromatographed on a 1 × 25-cm Ultrapore C_3 column (Beckman) equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1, vol/vol) and eluted at a flow rate of 2 ml/min, using the linear gradient depicted in Fig. 1*C*. The peak denoted by the arrow (Fig. 1*C*) was rechromatographed twice on a 0.46 × 25-cm Vydac 214 TP54 C_4 column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) and eluted at a flow rate of 1.5 ml/min, using the linear gradient depicted in Fig. 1 *D* and *E*.

Structural Characterization. Vapor-phase hydrolysis of the purified peptide (≈1 nmol) was carried out at 110°C for 24 hr with 5.7 M HCl. Amino acid compositions were determined by precolumn derivatization with phenyl isothiocyanate (26) using an Applied Biosystems model 420A derivatizer and model 130A separation system. The manufacturer's

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Abbreviations: NPY, neuropeptide Y; PTC, phenylthiocarbamoyl.
§To whom reprint requests should be addressed.

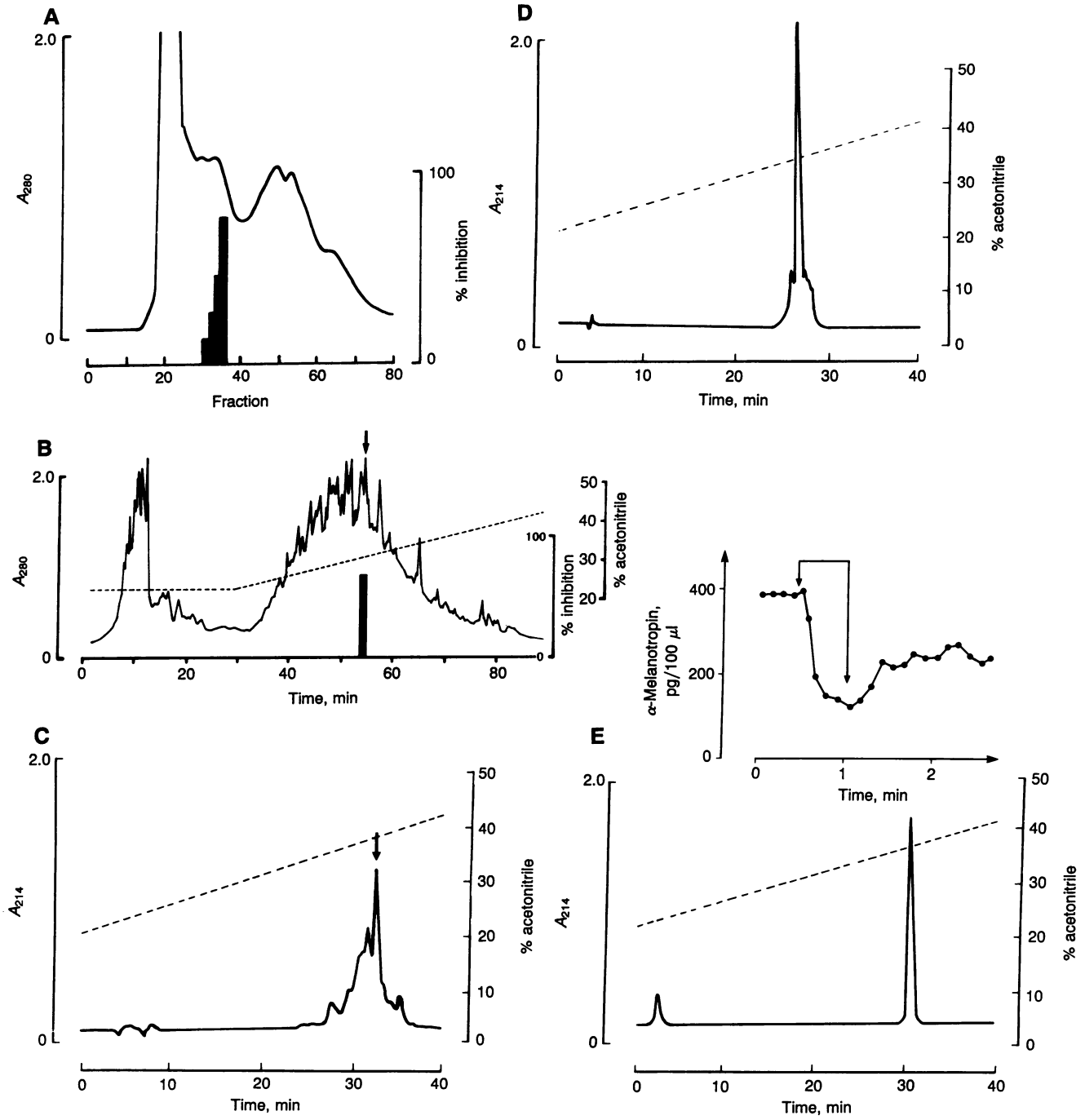

FIG. 1. Purification of melanostatin from frog brain. (*A*) The brain extract was purified by preparative gel filtration on a Sephacryl S-100 column and the fractions exhibiting melanostatin activity (% inhibition of α-melanotropin release; black bars) were pooled. (*B*) The active fractions were applied to a Vydac C_{18} column and the elution was carried out using a gradient of acetonitrile in 0.1% trifluoroacetic acid (broken line). The peak denoted by the arrow exhibited strong melanostatin activity (black bar). (*C*) The active fraction was rechromatographed on an Ultrapore C_3 column using a linear gradient of acetonitrile in 0.1% trifluoroacetic (broken line). (*D* and *E*) The peak material denoted by the arrow in Fig. 1*C* was chromatographed twice on a Vydac C_4 column using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. (*Inset*) Effect of the purified peptide on α-melanotropin release from perifused neurointermediate lobes. Downward vertical arrows indicate the duration (30 min) of the application of the peptide.

standard operating procedures were used and the detection limit for phenylthiocarbamoyl (PTC) amino acids was 0.5 pmol.

The primary structure of the peptide was determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin (PTH) amino acid under gradient elution conditions. Standard operating procedures were used and the detection limit for PTH amino acids was 0.5 pmol.

The presence of an α-amidated carboxyl-terminal tyrosine residue was investigated as described (27). Briefly, frog melanostatin (≈5 nmol) in 100 μl of 0.2 M ammonium bicarbonate buffer (pH 7.8) was incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) at an enzyme/substrate ratio of 1:50 for 16 hr at 37°C. The reaction mixture was lyophilized and redissolved in 20 μl of 1 mM EDTA. An aliquot (5 μl) was applied to the sintered glass disc of the 420A derivatizer and the PTC derivative of the carboxyl-terminal amino acid was

produced. The retention time on reverse-phase HPLC of the derivative was compared with the retention times of the PTC derivatives of tyrosine and L-tyrosinamide (Sigma).

Peptide Synthesis. Frog melanostatin was synthesized by the solid-phase method using an automatic Applied Biosystems model 430A synthesizer. The synthesis was carried out with a benzhydrylamine resin (28) (0.6 g; 0.40-mmol scale), since the peptide bore a carboxyl-terminal amide function. The peptide was assembled using the standard *t*-butyloxycarbonyl (Boc) chemistry, according to the Applied Biosystems standard procedure. The Boc-amino acids with appropriate side-chain protection were obtained from Richelieu Biotechnologies (St-Hyacinthe, Québec). The completed peptide was cleaved from the resin support and deprotected by treatment with HF (29).

After extraction from the resin and lyophilization, the peptide was purified by reverse-phase chromatography on a Waters DeltaPak column, using an eluent consisting of solvent A (0.06% trifluoroacetic acid) and solvent B (60% acetonitrile/0.06% trifluoroacetic acid). The peptide was eluted with successive linear gradients of solvent B. Analytical HPLC of the individual fractions was carried out and the fractions corresponding to the purified peptide were lyophilized. The purified material was characterized by analytical HPLC, capillary electrophoresis, and amino acid analysis.

Melanostatin Bioassay. Throughout the purification program, the melanotropin-release-inhibiting activity was assayed using the ability of a putative melanostatin to inhibit the release of α-melanotropin from perifused frog neurointermediate lobes as described (30). The concentration of α-melanotropin released in the effluent perifusate was assayed using a specific double-antibody radioimmunoassay (31).

RESULTS

The melanostatin-like activity in the frog brain extract was eluted from the Sephacryl S-100 gel permeation column in fractions 30–38 (Fig. 1*A*), suggesting that the bioactive peptide had a molecular weight of ≈4000. When the fractions from gel filtration were pooled and chromatographed on a C_{18} reverse-phase column, the melanostatin-like activity was associated with a single peak (Fig. 1*B*, arrow). The biologically active material was rechromatographed on a C_3 column, yielding several partially resolved peaks (Fig. 1*C*). The major component (arrow) was chromatographed on a C_4 column (Fig. 1*D*) and eluted as a sharp peak. This material was finally rechromatographed on a C_4 column (Fig. 1*E*). After this final step, the purity of the peptide was >98%, and the yield was ≈10.8 nmol. The purified peptide was highly active in inhibiting α-melanotropin release from perifused frog neurointermediate lobes (Fig. 1*E Inset*).

Amino acid analysis of the peptide indicated the probable composition $Asx_5Glx_3Ser_2Gly_1His_1Arg_3Thr_1Ala_4Pro_4Tyr_5Met_1Ile_2Leu_1Lys_2$. The structure of the peptide was determined by direct Edman degradation of 1 nmol of the intact peptide. The complete amino acid sequence of frog melanostatin is shown in Table 1. Since the penultimate carboxyl-terminal amino acid was arginine, tryptic mapping was carried out to determine whether the carboxyl-terminal tyrosine residue was amidated. The retention time of the PTC derivative of the carboxyl-terminal amino acid produced by trypsin digestion was 15.25 min. The retention times of tyrosine and tyrosinamide were 11.73 and 15.26 min, respectively.

A mixture of native frog melanostatin and its synthetic replicate possessing an amidated carboxyl terminus was chromatographed on a C_{18} HPLC column. The two peptides were eluted as a single sharp and symmetrical peak (data not shown). The synthetic replicate of melanostatin induced a dose-related inhibition of α-melanotropin release from whole frog neurointermediate lobes *in vitro* (IC_{50} = 60 nM; Fig. 2). The synthetic peptide also inhibited the generation of action potentials from frog melanotrophs in primary culture (data not shown).

Table 1. Automated Edman degradation of frog melanostatin

Cycle no.	Residue	Yield, pmol	Cycle no.	Residue	Yield, pmol
1	Tyr	451	19	Lys	122
2	Pro	389	20	Tyr	119
3	Ser	32	21	Tyr	142
4	Lys	363	22	Ser	12
5	Pro	263	23	Ala	88
6	Asp	272	24	Leu	112
7	Asn	243	25	Arg	107
8	Pro	230	26	His	129
9	Gly	126	27	Tyr	106
10	Glu	147	28	Ile	174
11	Asp	211	29	Asn	85
12	Ala	181	30	Leu	108
13	Pro	199	31	Ile	165
14	Ala	143	32	Thr	15
15	Glu	93	33	Arg	97
16	Asp	194	34	Gln	47
17	Met	122	35	Arg	93
18	Ala	140	36	Tyr	30

DISCUSSION

An efficient HPLC strategy was developed for the isolation of the peptide(s) exhibiting melanostatin activity in frog brain extracts. We here report the complete structural characterization of a 36-amino acid peptide with high intrinsic melanotropin-release-inhibiting activity. Frog melanostatin shows striking homology with peptides of the pancreatic polypeptide/peptide YY/neuropeptide Y family (Table 2). The only

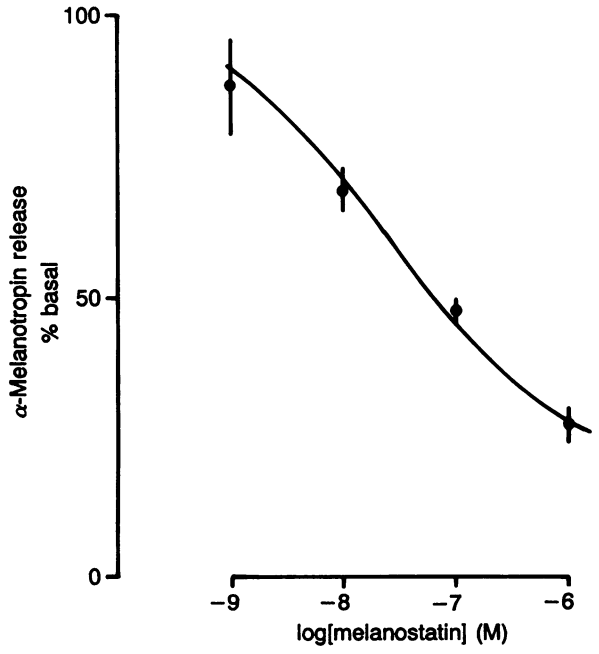

FIG. 2. Effect of increasing concentrations of synthetic frog melanostatin on α-melanotropin release from perifused frog neurointermediate lobes. The values were calculated from a series of data similar to that presented in Fig. 1*E Inset*.

Table 2. Comparison of the amino acid sequences of frog melanostatin and other members of the pancreatic polypeptide (PP)/peptide YY(PYY)/NPY family

Peptide	Amino acid sequence	Ref.
	1 10 20 30	
Frog melanostatin	YPSKPDNPGEDAPAEDMAKYYSALRHYINLITRQRYamide	
Human NPY	----------R----------amide	32, 33
Porcine PYY	--A--EA-----SP-ELSR--AS----L--V-----amide	34
Porcine PP	A-LE-VY--D--TP-Q--Q-AAE--R---ML--P--amide	35

structural difference between human NPY (32) and frog melanostatin is at position 19, where the arginine residue in human NPY is replaced by a lysine residue. This suggests that the peptide we have isolated actually corresponds to authentic frog NPY. This hypothesis is supported by physiological studies which showed that porcine NPY is a potent inhibitor of α-melanotropin release from pituitary melanotrophs in frog (18) and toad (19).

Biologically, frog melanostatin and the closely related mammalian NPY species exhibit many properties expected of a putative melanotropin-release-inhibiting factor. Besides their inhibitory effect on α-melanotropin secretion, these peptides suppress the electrical activity of pituitary melanotrophs (36) and reduce the formation of cAMP in frog pars intermedia (M.-C.T., J.-M.D., A. Enjalbert, and H.V., unpublished observations). Moreover, intra-aortic injection of synthetic NPY to freely moving *Xenopus* causes skin bleaching in black background-adapted animals (D. Boujard, J.-M.D., and H.V., unpublished work). These observations, together with the occurrence of a dense network of nerve fibers exhibiting NPY-like immunoreactivity in the pars intermedia of amphibians (18, 19), support the concept that the peptide isolated from frog brain tissue plays a physiological role in the regulation of pituitary melanotrophs.

It is not known whether, in mammals, NPY is also capable of inhibiting α-melanotropin release. However, there is ample precedent for conservation of the functions of hypothalamic neuropeptides in the vertebrate phylum (37–39). Various studies have established that pituitary melanotrophs are regulated by multiple factors, including classical neurotransmitters and neuropeptides (12, 30, 39–43). Coexistence of frog melanostatin with aminergic neuromediators and possible cooperation with other inhibitory factors, such as dopamine and γ-aminobutyrate, deserve further investigation.

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