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ISOLATION OF [PRO^2,MET^{13}]SOMATOSTATIN-14 AND SOMATOSTATIN-14 FROM THE FROG BRAIN REVEALS THE EXISTENCE OF A SOMATOSTATIN GENE FAMILY IN A TETRAPOD

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SUMMARY: Two somatostatin-related peptides were isolated in pure form from an extract of the brain of the European green frog, *Rana ridibunda*. The primary structure of the most abundant component was identical to that of mammalian somatostatin-14. The primary structure of the second component, present in approximately 5% of the abundance of somatostatin-14, was established as Ala-Pro-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Met-Cys. This sequence shows two substitutions (Pro for Gly^2 and Met for Ser^{13}) compared with mammalian somatostatin-14. The data provide evidence for a somatostatin gene family in tetrapods as well as in teleost fish.

The tetradecapeptide somatostatin has first been isolated from ovine hypothalamic extracts (1). Despite the fact that the complete amino acid sequence is not required for biological activity (2), the primary structure of somatostatin-14 has been remarkably well conserved during evolution of the vertebrates. The amino sequence of the peptide is the same in an agnathan [the Atlantic hagfish, *Myxine glutinosa* (3)], an elasmobranch [the ray, *Torpedo marmorata* (4)], several species of teleost fish [reviewed in (5)], an amphibian [the frog, *Rana pipiens* (6)], a reptile [the turtle, *Pseudemys scripta* (7)], a bird [the pigeon (8)] and all species of mammals yet studied. Prior to the present study, only two molecular variants of somatostatin-14 have been identified: [Ser^{12}]somatostatin-14 from the lamprey, *Petromyzon marinus* (9) and [Ser^5]somatostatin-14 from the holocephalan fish, *Hydrolagus colliei* (Pacific ratfish) (10).

Sequence analysis of DNA from a human (11) and a rat (12) λ phage library led to the identification of a single gene encoding prosomatostatin (prosomatostatin I) in mammals. However, cloning and sequence analysis of DNAs complementary to mRNAs from islet tissue from the teleost fish, *Lophius americanus* (anglerfish) suggested the possibility of a somatostatin gene family (13). A second somatostatin precursor (prosomatostatin II), which terminated in the sequence [Tyr^7,Gly^{10}]somatostatin-14, was identified in addition

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of prosomatostatin I. Subsequent work has led to the isolation of peptides derived from the post-translational processing of prosomatostatin II from the endocrine pancreas of several teleost species. The precursor is not processed to a tetradecapeptide but to larger somatostatin-related peptides of between 25 and 28 amino acid residues [reviewed in (5)]. At the present time, there is no evidence for the expression of prosomatostatin II in species other than teleost fish and the existence of multiple somatostatin genes in other classes of vertebrates has not been conclusively demonstrated. The present study shows for the first time that the frog brain contains two distinct somatostatin-related peptides that are probably derived from different biosynthetic precursors.

MATERIALS AND METHODS

Preparation of frog brain extract

The extraction of whole brain (94.5 g) from 1200 specimens of adult *Rana ridibunda* has been described previously (14). Peptide material was isolated from the extract by using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.). Bound material was eluted with acetonitrile/water/ trifluoroacetic acid (70:29:1, by vol.) and freeze-dried.

Purification of frog somatostatin-related peptides

The brain extract, after partial purification on Sep-Pak cartridges, was chromatographed on a Sephacryl S-100 (Pharmacia, Uppsala, Sweden) column as previously described (14). Fractions with K_{AV} between 0.8 and 1.0, previously shown to contain α- and γ-melanocyte-stimulating hormone (15) were pooled and pumped on to a Vydac 218TP54 reversed-phase C_{18} HPLC column (1 cm x 25 cm) (Separations Group, Hesperia, CA, U.S.A.) equilibrated with 0.1% (v/v) trifluoroacetic acid at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min, held at this concentration for 30 min and then raised to 49% (v/v) over 60 min with linear gradients. Absorbance was measured at 214 nm and 280 nm and individual peaks were collected by hand. The peak designated S-I (Fig. 1) was rechromatographed on a Vydac 214TP54 reversed-phase C_4 column (0.46 cm x 25 cm) equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1, by vol.) at a flow rate of 1.5 ml/min. The concentration of acetonitrile was raised to 38% (v/v) over 50 min using a linear gradient. The peak designated S-II was chromatographed on the C_4 column under the same conditions of elution except that the concentration of acetonitrile was raised to 42% (v/v) over 40 min. Peptides S-I and S-II were purified to apparent homogeneity by chromatography on a Vydac 219TP54 phenyl column (0.46 cm x 25 cm) equilibrated with the same solvent used for the C_4 column. The concentration of acetonitrile was raised from 21% (v/v) to 42% (v/v) over 40 min.

Structural characterization of frog somatostatin-related peptides

Frog somatostatins S-I and S-II (approx. 1 nmol) were reduced (with dithiothreitol) and pyridylethylated (with 4-vinylpyridine) as previously described (7). The derivatized peptides were purified on a Vydac C_4 column under the condition shown in Fig. 2. The primary structures of the pyridylethylated peptides were determined by automated Edman degradation in an Applied Biosystems model 471A Sequenator modified for detection of amino acid phenylthiohydantoin derivatives under gradient elution conditions. The detection limit for phenylthiohydantoin derivatives was 1 pmol. Amino acid compositions were determined by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer (Foster City, CA), followed by separation of the phenylthiocarbamyl amino acids by reversed phase HPLC (16). Hydrolysis in 5.7 M hydrochloric acid (24 h at 110 C) of approximately 500 pmol of peptide was carried out. Cysteine and tryptohan residues were not determined.

RESULTS

Peptide purification

The elution volume on a semi-preparative C_{18} reversed-phase column of the extract of frog brain, after partial purification on a Sephacryl S-100 column, is shown in Fig. 1. As described previously (15), a total of 93 peaks, containing peptides in the 1000 - 2500 molecular mass range, were collected and subjected to further purification on analytical reversed-phase columns. Peptide S-I, subsequently shown to be

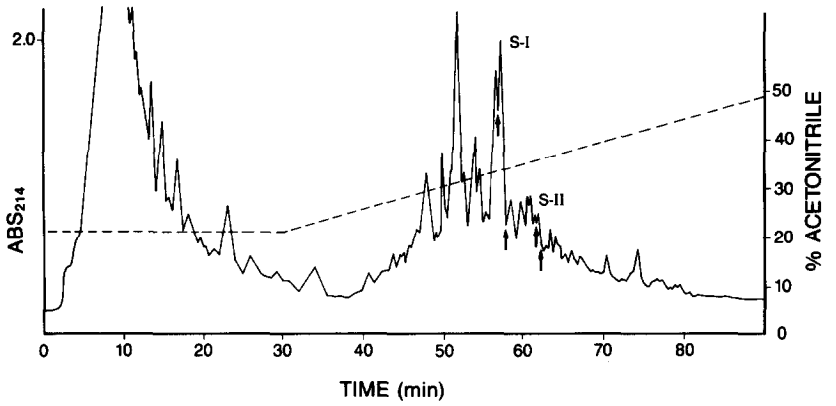

Fig. 1. Reversed-phase HPLC on a semi-preparative Vydac C_{18} column of an extract of frog brain after initial fractionation on a Sephacryl S-100 column. Peptides in the molecular mass range 1000-2500 were selected for HPLC purification. The peak designated S-I contains somatostatin-14 and peak S-II contains [Pro^2,Met^{13}]somatostatin-14. The broken line shows the concentration of acetonitrile in the eluting solvent and the arrows show where peak collection began and ended.

somatostatin-14 and peptide S-II, subsequently shown to be [Pro^2,Met^{13}]somatostatin-14, were eluted from a C_4 column as sharp symmetrical peaks (Fig. 2). The peptides were also eluted from an analytical Vydac phenyl column as single sharp peaks (chromatograms not shown). The final yields of pure peptides, as determined by amino acid analysis, were: S-I, 24 nmol and S-II, 1.4 nmol.

Structural characterization

The amino acid composition of peptide S-I (found: Asx 1.1, Ser 1.2, Gly 1.2, Thr 2.1, Ala 1.2, Phe 3.0, Lys 2.0 residues/mol peptide) indicated that the component was probably identical to somatostatin-14. The composition of peptide S-II (found Asx 1.1, Thr 2.0, Ala 1.1, Pro 1.1, Met 0.9, Phe 2.7, Lys 1.8) indicated

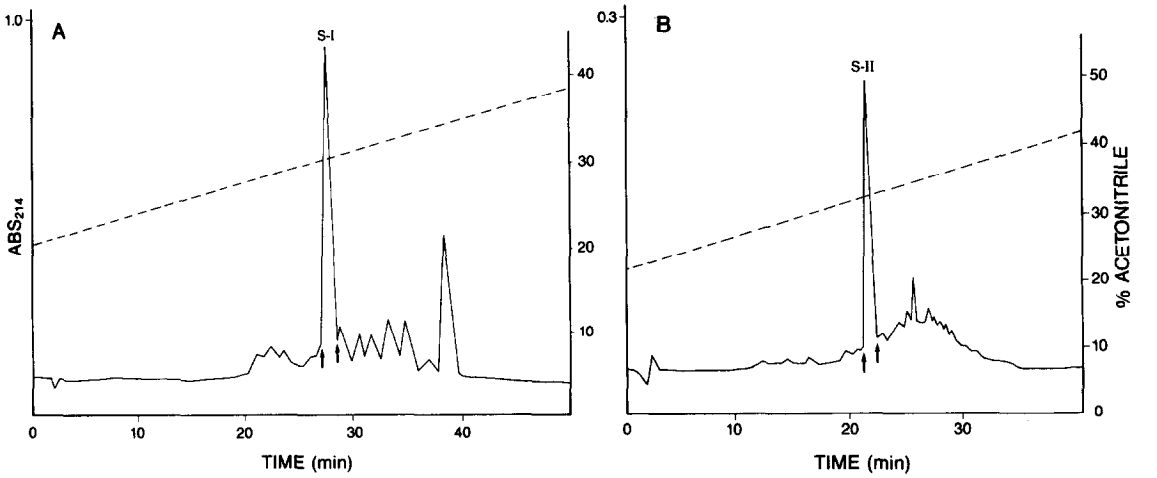

Fig. 2. Reversed-phase HPLC on a Vydac C_4 column of (A) peptide S-I (somatostatin-14) and (B) peptide S-II [Pro^2,Met^{13}]somatostatin-14. Details of the elution conditions are given in the text.

TABLE 1. Determination of the primary structures of somatostatin-related peptides from frog brain by automated Edman degradation

Cycle no.	Peptide S-I Amino acid	Yield (pmol)	Peptide S-II Amino acid	Yield (pmol)
1	Ala	767	Ala	434
2	Gly	858	Pro	239
3	PE-Cys	412	PE-Cys	182
4	Lys	705	Lys	188
5	Asn	531	Asn	153
6	Phe	780	Phe	262
7	Phe	854	Phe	276
8	Trp	152	Trp	48
9	Lys	248	Lys	98
10	Thr	71	Thr	31
11	Phe	345	Phe	102
12	Thr	67	Thr	28
13	Ser	33	Met	33
14	PE-Cys	29	PE-Cys	11

PE-Cys refers to the vinylpyridine derivative of cysteine.

that S-II contained additional proline and methionine residues but lacked serine and glycine compared with somatostatin-14. The results of Edman degradation are shown in Table 1. It was possible to identify amino acid phenylthiohydantoin derivatives without ambiguity and the data confirm that peptide S-I is identical to somatostatin-14 and demonstrate that peptide S-II contains the substitution Pro for Gly at position 2 and Met for Ser at position 13.

DISCUSSION

This study has led to the isolation from amphibian nervous tissue of a novel molecular variant of somatostatin, [Pro^2,Met^{13}]somatostatin-14, and thus provides strong evidence for the existence of a somatostatin gene family in a tetrapod. We confirm the observation of Takami et al. (6) that the predominant molecular form of frog somatostatin-14 is identical to mammalian somatostatin-14. It is not clear, however, whether the somatostatin-14 variant was absent from the brain of the species of frog studied by these workers (*Rana pipiens*) or was present but not detected. An understanding of the biosynthetic relationship between the two molecular forms of *Rana ridibunda* somatostatin requires the determination of the nucleotide sequence of the genes (or corresponding cDNAs) encoding the precursors of the peptides. It has been speculated that prosomatostatin I and II in the islet tissue of the anglerfish arose from duplication of an ancestral gene (13). The substitution Met for Ser and Pro for Gly in the two frog somatostatins cannot be accomplished by single nucleotide changes in the somatostatin gene and these particular substitutions are not found in an alignment of the amino acid sequences of anglerfish prosomatostatin I and II (13).

The existence of different molecular forms of somatostatin-14 in amphibia raises the possibility that multiple somatostatin gene products are present in other classes of vertebrate. Four peptides with somatostatin-like ability to inhibit the release of growth hormone from anterior pituitary cells were identified in an extract of chicken hypothalamus (17). The amino acid sequences of these peptides were not determined but compositional analysis indicated that one component represented mammalian somatostatin-14, one

component probably represented [Leu^{8}]somatostatin-28, a third component had the same composition as somatostatin-14 but different chromatographic properties and the fourth peptide contained additional tyrosine and arginine residues. Similarly, although only one rat somatostatin gene was unambiguously identified by Tavianini et al. (12) in a λ phage gene library, Southern hybridization analysis using a somatostatin-14 cDNA probe indicated that the genome may contain a second region with structural similarity to the characterized somatostatin gene.

The distribution of somatostatin-containing neurons and somatostatin receptors in the brain of *Rana ridibunda* has been studied in detail by Laquerriere et al. (18). There was good correlation between the localization of somatostatin-immunoreactive perikarya and fibers and somatostatin-binding sites in several brain regions e.g. the median pallidum, tectum and interpeduncular nucleus but pronounced mismatching in other regions e.g. the olfactory bulb, lateral pallium, cerebellum and hypothalamic areas. In the anglerfish islet, immunohistochemical studies with specific antisera have shown that prosomatostatin I and II genes are expressed in different populations of cells (19). Clearly, the study of Laquerriere et al. (18) must now be extended by using antisera specific for [Pro^{2},Met^{13}]somatostatin-14 to determine the distribution of neurons that synthesize this component and using a radioiodinated derivative of the peptide to determine the location of specific binding sites.

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