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Isolation and structure of pro-somatostatin: A putative somatostatin precursor from pig hypothalamus

(amino acid sequence/pro-hormone)

ANDREW V. SCHALLY*, WEI-YONG HUANG*, ROBERT C. C. CHANG*, AKIRA ARIMURA*, TOMMIE W. REDDING*, ROBERT P. MILLAR*†, M. W. HUNKAPILLER‡, AND L. E. HOOD‡

*Endocrine and Polypeptide Laboratory, Veterans Administration Medical Center, and Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana 70146; and ‡Division of Biology, California Institute of Technology, Pasadena, California 91125

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ABSTRACT **An octacosapeptide that we named pro-somatostatin has been isolated from acid extracts of porcine hypothalami and found to have the amino acid sequence Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys. This octacosapeptide possesses high somatotropin (growth hormone) and prolactin release-inhibiting activity *in vitro*. It also crossreacts strongly with antisera generated against the somatostatin tetradecapeptide. This octacosapeptide is most likely a precursor (pro-hormone) of somatostatin in the hypothalamus. The existence of still larger molecular size precursors of somatostatin was also observed.**

More than 5 years ago, our laboratory reported larger, more basic forms of somatostatin in fractions of pig hypothalamic extract (1, 2). We also recognized two types of immunoreactive somatostatin in extracts of rat pancreas, stomach, and duodenum (3). We suggested, on the basis of physicochemical, biological, and immunological data, that these substances represented precursors of somatostatin (1–3). Later, we postulated that the hypothalamic pro-somatostatin has an extension at the NH_2 terminus containing one or more arginine residues (4–6). Manual degradation by the Edman–dansyl method showed the NH_2 terminal sequence to be Ser-Ala-Asn-Ser. However, because of the small amounts available, we were unable to complete the entire sequence. Recent developments in automated amino acid sequence analysis (7), which led to an increase in sensitivity of several thousandfold, permitted the successful elucidation of the whole sequence. In this paper we describe the isolation, biological and immunological characterization, and structural elucidation of putative pro-somatostatin from porcine hypothalami. The sequence of hypothalamic pro-somatostatin is identical with that of a putative precursor of somatostatin from pig intestine recently reported by Pradayrol *et al.* (8). We conclude that in the hypothalamus, as in the gut, somatostatin also exists in the form of the pro-hormone.

MATERIALS AND METHODS

Purification. A total of 630,000 pig hypothalami was dissected, defatted, and extracted as described (9, 10). The major part of this extract, corresponding to 470,000 hypothalami, was the same as that used for the isolation of porcine somatostatin (2) and pro-[Met]enkephalin (11). This extract was purified first by gel filtration on Sephadex G-25 (9, 10). Fractions containing immunoreactive somatostatin from the gel filtration were extracted with the upper phase of 0.1% acetic acid/1-butanol/pyridine, 11:5:3 (vol/vol), (system I) and lyophilized. This material was then further purified by preparative countercurrent distribution (CCD) in the same system by the C-2 model apparatus (H. O. Post, New York), followed by chromatography on carboxymethyl (CM)-cellulose, another CCD in system I for 1800 transfers in the A-4 apparatus, chromatography on SE-Sephadex, analytical gel filtration on Sephadex G-25, and rechromatography on SE-Sephadex. A smaller part of this extract, corresponding to 160,000 hypothalami, was purified by similar methods except that free-flow electrophoresis and CCD in 1-butanol/acetic acid/water, 4:1:5 (vol/vol), were used instead of other steps. The final purification was done by high-pressure liquid chromatography (HPLC) (11). In all the steps, the separation pattern was followed by the Folin–Lowry reaction (12), by absorbance readings at 278 nm or, for HPLC, at 220 and 280 nm. All the extraction and purification procedures and most methods used for determination of homogeneity and composition and for manual Edman–dansyl degradation have been described (2, 9–11). Automated amino acid sequence analysis was performed on a spinning cup sequenator designed and built at California Institute of Technology (7). The sequenator program for the Edman degradation was similar to that described (13). It included a 25-min coupling step with 0.17 M Quadrol buffer, a single 4-min cleavage step, and automated conversion of the anilinothiazolinones to phenylthiohydantoin amino acids by treatment with 25% (vol/vol) aqueous trifluoroacetic acid for 40 min. The spinning cup and conversion flask were maintained at 52°C. Polybrene (Aldrich, 6 mg) and glycylglycine (100 nmol), dissolved in 0.5 ml of distilled water, were placed in the sequenator cup and subjected to eight complete degradation cycles. The pro-somatostatin (2 μg), dissolved in 0.5 ml of 10% (vol/vol) acetic acid, was then inserted and the sequencing program was restarted at the coupling stage. On cycles 6 and 10, the cleavage step was extended to 10 min to prevent excessive overlap due to incomplete removal of the prolyl residues (14). Phenylthiohydantoin amino acids were identified by HPLC on a Dupont Zorbax CN column. Details of identification of phenylthiohydantoin amino acids by this method and standard chromatograms have been described (15).

Bioassays for Pro-Somatostatin Activity. The inhibition of the release of somatotropin (growth hormone; GH) *in vitro* from isolated rat pituitary halves was measured as described (16). In some experiments, the inhibition of release of GH and prolactin was measured by using dispersed rat pituitary cells in monolayer cultures (17). Each sample was assayed in qua-

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Abbreviations: CCD, countercurrent distribution; CM-cellulose, carboxymethylcellulose; HPLC, high-pressure liquid chromatography; GH, somatotropin (growth hormone); RIA, radioimmunoassay.

† Present address: Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, Cape Town, South Africa.

druplicate. The concentration of GH and prolactin in the medium was measured by respective radioimmunoassays (RIAs) (16, 18) with the National Institute of Arthritis, Metabolism and Digestive Diseases kits.

RIA for Somatostatin. The concentration of porcine pro-somatostatin activity in various fractions was also followed by a RIA method for somatostatin (6, 19).

RESULTS

Isolation and Amino Acid Composition. After gel filtration of 2 M acetic acid extracts from 470,000 pig hypothalami (lyophilized weight, 3.6 kg) on a preparative column of Sephadex G-25 (2, 9, 10), immunoreactive somatostatin was found in several fractions. However, about 75% of total RIA activity in the extract was in fractions 657–856 with R_F = 0.7–0.53 (elution volume/total volume = 0.48–0.63) (see figure 1 of ref. 2). The total yield of immunoreactive somatostatin from 44 columns was 14.1 mg in terms of somatostatin tetradecapeptide as assayed by the RIA.

The Sephadex concentrate (382 g) was dissolved in 2800 ml of the lower phase of system I and extracted five times with the upper phase. After lyophilization of the combined upper phase, 38 g of extract was obtained, which contained 2.4 mg of immunoreactive equivalents of somatostatin or 17% of the total. The lower phase residue weighed 380 g and contained 11.8 mg of immunoreactive somatostatin. The extract inhibited GH release *in vitro* in doses of 40 μg/ml and the residue in doses of 500 μg/ml.

The upper phase extract was subjected to 250 transfers by CCD in system I. As reported (2), this procedure completely separated porcine somatostatin found in fractions 140–180 (K = 1.7) from pro-somatostatin fractions with a mean K = 0.07 (K is the partition coefficient). These pro-somatostatin fractions weighed 6.8 g and were equivalent to 900 μg of immunoreactive somatostatin. These fractions, in doses of 8 μg/ml, also inhibited GH release from isolated pituitary halves by 50% or more (Table 1).

Materials with pro-somatostatin activity from CCD I were purified by ion-exchange chromatography on a CM-cellulose column. The separation pattern can be seen in Fig. 1. Immunoreactive pro-somatostatin activity appeared in fractions 331–348, with a conductivity of 18.0–20.6 mS. These fractions also inhibited GH release *in vitro* from pituitary halves in doses of 1 μg/ml (Table 1). These fractions were combined, yielding 119 mg. Because only about one-third of immunoreactivity was recovered, a great loss of pro-somatostatin occurred in this step.

Table 1. Effect of porcine pro-somatostatin on release of GH from rat pituitaries

Purification stage	Dose dry wt, μg/ml	Medium GH, % of control*	P vs. control
Control	—	91.7 ± 3.6	—
CCD I	8	38.5 ± 3.8	0.01
Control	—	91.6 ± 3.4	—
CCD I	2	47.4 ± 12	0.01
Control	—	111.7 ± 12.7	—
Free-flow electrophoresis	2	52.6 ± 6.6	0.01
Control	—	136.8 ± 9.9	—
CM-cellulose	1	79.8 ± 2.6	0.01
Control	—	119 ± 7.4	—
CCD II	0.2	71 ± 6.5	0.01
Control	—	107.9 ± 1.8	—
SE-Sephadex II	0.2	56.0 ± 7.1	<0.01

* (2nd hr/1st hr) × 100. SEM are shown.

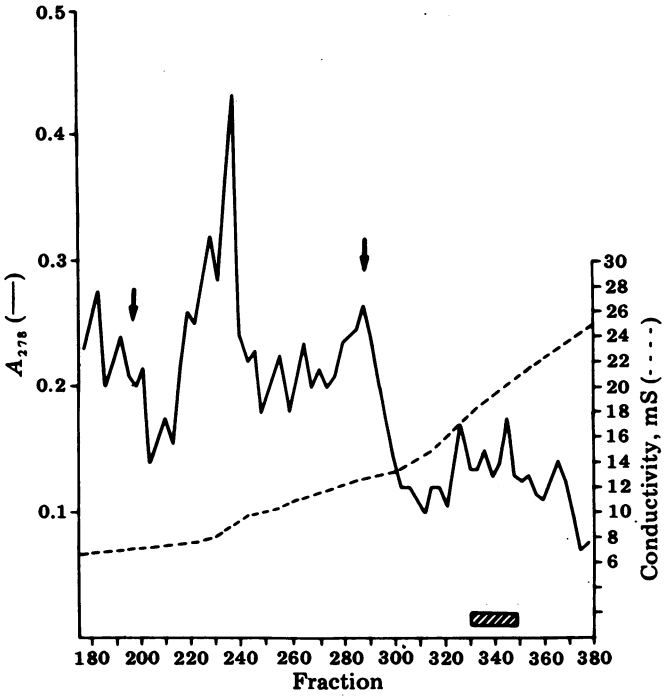

FIG. 1. Chromatography of 6.8 g of pro-somatostatin concentrate from CCD I on a CM-cellulose column (2.6 × 68 cm) equilibrated with 2 mM ammonium acetate buffer (pH 4.5). Gradient 1, to pH 7, 0.1 M buffer, through a 2000-ml mixing flask, was started at fraction 26; gradient 2, to pH 7, 0.25 M buffer was applied at fraction 197; gradient 3, to pH 7, 0.5 M buffer was started at fraction 288. Fraction size was 25 ml. Hatched bar at bottom indicates immunoreactive somatostatin.

The fractions from CM-cellulose were subjected to 1800 transfers by CCD in system I (Fig. 2). The RIA for somatostatin showed the presence of immunoreactive material in fractions 220–399 (mean K = 0.12). These fractions weighed 42.8 mg and contained 5.9 ng of pro-somatostatin per μg of RIA or a total of 253 μg. The same fractions also powerfully inhibited GH release *in vitro* in doses of 0.2 μg dry weight/ml (Table 1).

The active fractions were repurified by chromatography and rechromatography on SE-Sephadex. The separation pattern for the rechromatography can be seen in Fig. 3. Fractions 139–150, with a conductivity of 16.8–17.2 mS, contained pro-somatostatin activity and were lyophilized to yield 2.4 mg of material.

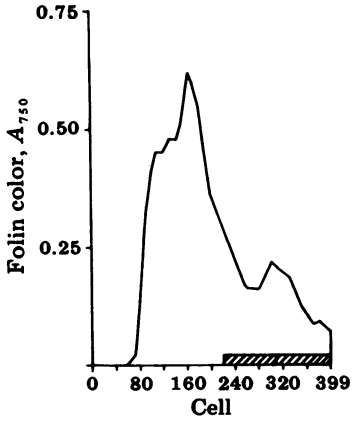

FIG. 2. CCD II of 119 mg of pro-somatostatin from CM-cellulose in system I. Lower phase was 3 ml; upper phase, 5 ml. The number of transfers was 1800. Folin–Lowry analyses were done on 50-μl aliquots of lower phase (cells 0–399). Hatched bar at bottom indicates immunoreactive somatostatin.

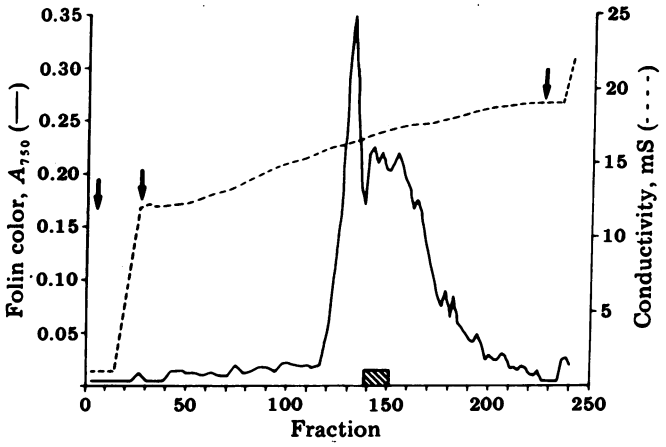

FIG. 3. Rechromatography of pro-somatostatin on an SE-Sephadex column (1.5 × 140 cm), equilibrated with 0.75 M pyridine acetate buffer (pH 5.3). Fraction size, 10 ml. A linear gradient to 1.8 M buffer (pH 5.3) was started at fraction 28. Hatched bar at bottom indicates somatostatin immunoreactivity.

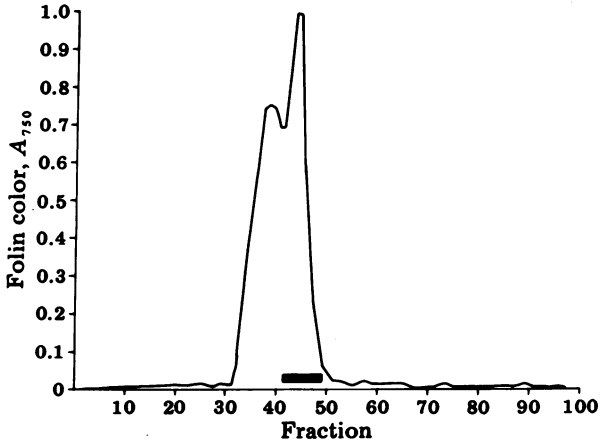

FIG. 4. Gel filtration of 2.4 mg of pro-somatostatin from SE-Sephadex on Sephadex G-25 (0.9 × 148 cm). Holdup volume, 28 ml; solvent, 0.2 M acetic acid; fraction size, 1.0 ml. Folin–Lowry analyses were done on 50-μg aliquots. Hatched bar at bottom indicates immunoreactive somatostatin.

The content of pro-somatostatin was 88 ng/μg dry weight, equivalent to a total of 211 μg. These fractions, in doses of 0.2 μg/ml, strongly inhibited GH secretion from rat pituitaries (Table 1) and also caused a decrease to one-seventh and one-half the original value, respectively, in GH and prolactin release from monolayer cultures of rat pituitary cells (Table 2).

The next purification step consisted of rechromatography on an analytical column of Sephadex G-25 (Fig. 4). Pro-somatostatin emerged in fractions 41–50 with an R_F = 0.6. After lyophilization, 1.9 mg of material was obtained with a somatostatin immunoreactivity of 95 ng/μg dry weight. Rechromatography on SE-Sephadex did not significantly increase the specific immunoreactivity of this fraction, which also inhibited GH and prolactin release from monolayer cultures in doses as low as 0.05 μg/ml (Table 2).

This pro-somatostatin (1.1 mg) was combined with another batch of material (dry weight, 1.5 mg) with equivalent somatostatin immunoreactivity (100 ng/μg) and biological activity, purified by similar methods from 160,000 hypothalami except that free-flow electrophoresis (Table 1) and CCD in 1-butanol/acidic acid/water, 4:1:5 (vol/vol), were used instead of extraction with solvent I. The amino acid composition of both materials was similar to that of somatostatin except that additional residues of aspartic acid (two), proline (two), serine (two), alanine (two), valine (one), lysine (one), and arginine (three) were also detected. This combined material (3.4 mg dry weight), which contained 280 μg of immunoreactive pro-somatostatin as determined by RIA for somatostatin, was subjected to HPLC in various systems in batches of 300 μg in order to determine the best separation conditions. To minimize potential adsorption losses on glass, we used polypropylene tubes for collection of fractions in all HPLC steps. Nevertheless, major losses of pro-somatostatin occurred in HPLC during either chromatography or flash evaporation and lyophilization, where contact with glass was unavoidable. After pro-somatostatin activity was located, the combined active fractions were reprocessed by HPLC. The pattern obtained on HPLC is shown in Fig. 5. Somatostatin immunoreactivity was found in both peak I, with a retention time of 40.9 min, and peak II, with a retention time of 42 min. These peaks were only partially separated. Because at this stage the dry weight could be unreliable, the total amount of material was also estimated from amino acid analysis after hydrolysis. Peak I contained 400 μg dry weight—148 μg of peptide material and 47 μg of somatostatin equivalents by RIA. Peak II had 390 μg dry weight—about 130 μg of peptide; its immunoreactivity was equivalent to 65 μg of somatostatin or 166 ng/μg dry weight. Portions of both peaks were used for composition and structural work. The remaining materials from both peaks were repurified separately by HPLC.

The HPLC elution profile of 24-μg aliquots of immunoreactive material from peak II is illustrated in Fig. 6. The somatostatin immunoreactivity emerged in fractions with a retention time of 17–33 min. This fraction contained 16 μg of peptide and 7.4 μg of immunoreactive somatostatin. An aliquot of 1.2 μg of peptide from this peak was subjected to amino acid analysis, which gave the following molar ratios: Asp, 3.2; Thr, 1.9; Ser, 2.6; Glu, 1.3; Pro, 1.9; Cys, 2.0; Gly, 1.2; Ala, 3.6; Met, 1.0; Phe, 2.8; Lys, 2.9; Arg, 1.6; and Trp, 1.0.

Table 2. Effect of porcine pro-somatostatin on release of GH and prolactin from rat pituitary cells in monolayer cultures

Purification stage	Dose dry wt, μg/ml	Medium GH,* ng/ml	Medium prolactin,* ng/ml	*P* vs. control
Control	—	2560 ± 80	—	—
1st SE-Sephadex	0.2	230 ± 50	—	<0.01
Control	—	2560 ± 80	2340 ± 34	—
2nd SE-Sephadex	0.2	350 ± 30	1800 ± 18	<0.01
Control	—	1288 ± 48	755 ± 31	—
3rd SE-Sephadex	0.05	752 ± 125	360 ± 56	<0.01

* SEM are shown.

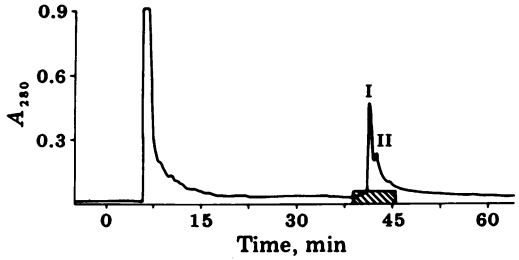

FIG. 5. Reverse-phase HPLC of pro-somatostatin. Solvent A, 10% (vol/vol) CH_3CN in 10 mM ammonium acetate (pH 4.0). Solvent B, 90% (vol/vol) CH_3CN in 10 mM ammonium acetate (pH 4.0) plus 1% *N*-ethylmorpholine. The peptide was applied to a μ-Bondapak C_{18} column (0.4 × 30 cm) and eluted isocratically at 0.5 ml/min with 20% (vol/vol) solvent B for 20 min, then by a linear gradient to 30% (vol/vol) solvent B at 25°C. Hatched bar at bottom indicates somatostatin immunoreactivity.

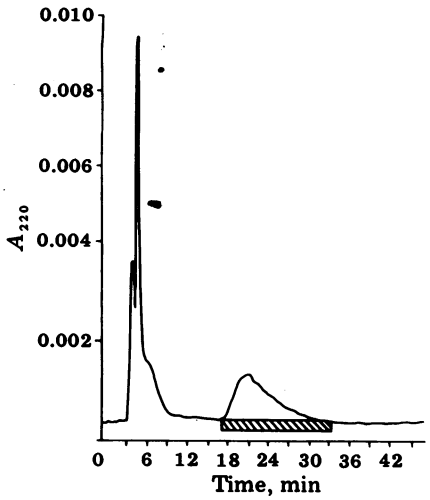

FIG. 6. Reverse-phase HPLC of pro-somatostatin peak II from HPLC. The peptide (24 μg) immunoreactive material was applied to a μ-Bondapak C_{18} column (0.4 × 25 cm) and eluted at 0.5 ml/min with 2-propanol/10 mM ammonium acetate, pH 4.0, by using a linear gradient of 2-propanol from 20% to 50% for 50 min at 25°C. Hatched bar at bottom indicates somatostatin immunoreactive fractions.

Sequential Analyses. Although fractions from peak I of Fig. 5 had higher peptide content, no end group could be detected by the dansyl procedure. Similarly, automated Edman degradation did not lead to the release of any phenylthiohydantoin amino acids. Therefore, fractions from Fig. 6 were selected for amino acid sequence determination. An aliquot of 2 μg of peptide was subjected to manual sequential analyses by the combined Edman–dansyl procedure for four steps. Another portion of 2 μg was digested with aminopeptidase M, followed by the dansyl reaction. On the basis of these two experiments, the NH_2-terminal sequence was identified as Ser-Ala-Asn-Ser.

Another 2-μg aliquot of the same fractions was subjected to automated sequence analysis in the Caltech sequenator. This allowed assignment of 26 amino acid residues as follows:

Ser-Ala-Asn*-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu*-Arg-Lys-

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

Residues were identified by: —, two-dimensional thin-layer chromatography of dansyl amino acids; —, automated amino acid sequence determinations (sequenator) and high-sensitivity HPLC analysis of phenylthiohydantoin amino acids (figures on identification of phenylthiohydantoin amino acids are available from the authors); and *, aminopeptidase M digestion and amino acid analysis. The assignments agreed with the amino acid composition of the peptide except for the absence of two residues of Cys. Yields of phenylthiohydantoin-Cys from the sequenator at the levels of peptide analyzed in this experiment were not sufficient for direct identification. However, the absence of assignment of any other phenylthiohydantoin amino acids at cycles 17 and 28 places the Cys residues at these positions. The proposed primary structure of the pro-somatostatin is that of the octacosapeptide shown.

Biological and Immunological Characterization. The extremely small amounts of natural pro-somatostatin octacosapeptide available precluded an extensive evaluation of its activities in view of the wide variety of endocrine and exocrine effects exerted by somatostatin tetradecapeptide. Natural pro-somatostatin inhibited GH and prolactin release from monolayer cultures of rat pituitary cells. Its activity was about half that of somatostatin on a weight basis. Natural pro-somatostatin was indistinguishable immunologically from synthetic pro-somatostatin, as indicated by the parallelism of dilution curves in the RIA system. The slope of the dilution curve for these pro-somatostatin preparations ($b = -1.82$) was less steep than that for synthetic somatostatin ($b = -2.51$), indicating less affinity to antiserum A 101 generated against somatostatin tetradecapeptide, the recognition sites of which are directed toward the Asn^5 to Ser^{13} portion of the molecule, included in the ring structure (6).

DISCUSSION

This paper reports the isolation and structure of a putative pro-somatostatin from porcine hypothalami. This octacosapeptide proved to be an NH_2-terminally extended form of somatostatin.

We detected the presence of very basic, higher molecular weight, immunoreactive forms of somatostatin in pig hypothalami more than 5 years ago and suggested that they might represent precursors of somatostatin (1–5). For instance, on the basis of a large number of CCD distributions, *K* of pro-somatostatin in solvent I was 0.12 and in solvent II (1-butanol/acetic acid/water, 4:1:5) it was 0.07, as compared with respective *K* values for somatostatin of 1.7 and 0.6. We also reported that the octacosapeptide with somatostatin activity is also much more basic than somatostatin; it was eluted from CM-cellulose at 19 mS and from SE-Sephadex at 17 mS, as opposed to somatostatin for which these values are 11.8 mS and 12 mS, respectively. Immunological studies (6) using various antisera with different recognition sites indicated that porcine pro-somatostatin is an NH_2-terminally extended form of somatostatin. However, because only small amounts of highly purified material were available, elucidation of the complete structure was delayed. Subsequently, other laboratories confirmed the presence of these higher molecular weight forms of somatostatin in rat (20), pig (21), sheep (22–24), and mouse (25) hypothalamus.

It was also thought that other presumptive precursors of hypothalamic somatostatin had a large molecular weight (1). It is probable that part of immunoreactive somatostatin in the residue remaining in the lower phase of system I represents this very large molecular weight form of pro-somatostatin (2) because much of octacosapeptide pro-somatostatin, as well as all of somatostatin, must have been extracted with the upper phase.

Large forms of immunoreactive somatostatin were also previously reported by us in the pancreas, stomach, and duodenum of rats (3). Subsequently, other investigators reported and partially characterized larger immunoreactive forms of somatostatin in rat (20, 26), dog (27), angler fish (28), and pigeon (29) pancreas, rat (20), and dog (30) stomach, and human (31) pancreatic somatostatinoma. Larger forms of immunoreactive somatostatin were also detected in human serum (32) and rat plasma (33).

Some of these big forms of somatostatin could be dissociated by urea into smaller fragments, suggesting that they were not covalently bound and, therefore, were not authentic higher molecular weight species (26). Other forms of big immunoreactive pro-somatostatin could be dissociated only after treatment with disulfide bond-reducing agents (27–30), suggesting that somatostatin may be covalently bound by disulfide bonds to larger polypeptides.

Recently, Pradayrol *et al.* (8) reported the complete structure of a putative pro-somatostatin from porcine upper small in-

testine. The structure of pro-somatostatin isolated by us from the hypothalamus is identical to that from the gut (8). Although the concentration of total immunoreactive somatostatin is higher in the hypothalamus than in the intestine, the total content of somatostatin in the intestine is much greater because of the large size of this organ. From the work of Pradayrol *et al.* (8, 34) and our work, it is likely that somatostatin is formed by cleavage of the Lys-Ala peptide bond in the octacosapeptide pro-somatostatin. However, a demonstration of the conversion of the putative pro-somatostatin to somatostatin by the tissues of origin or appropriate subcellular fractions is necessary to identify the octacosapeptide as a pro-somatostatin. It is not clear if the 28-amino-acid octacosapeptide pro-somatostatin is cleaved post-translationally from a still larger prohormone, but its existence in both the hypothalamus and the gut suggests that octacosapeptide could be one of the intermediate physiological precursors of somatostatin stored in those organs.

The detection of a putative precursor of hypothalamic and intestinal somatostatin agrees with the previously established existence of pro-forms of peptide hormones gastrin, insulin, glucagon, parathyrin, β-melanotropin, and β-endorphin (8, 35). The sequences of regions adjacent to cleavage sites for conversion of all these peptide pro-hormones invariably contain two consecutive basic residues (Lys-Lys, Arg-Arg, Lys-Arg, or Arg-Lys), suggesting that they might be split by trypsin-like proteolytic enzymes with the resulting formation of the respective hormones. A high frequency of serine and proline residues was also observed in the precursors. The primary structure of presumptive pro-somatostatin is in excellent agreement with these findings because it contains an Arg-Lys dipeptide in positions immediately preceding the somatostatin sequence as well as two serine and two proline residues in the NH_2-terminal extension.

The octacosapeptide pro-somatostatin possesses considerable biological and immunological activity. Its GH and prolactin release-inhibiting activity *in vitro* is about 50% of that of somatostatin on a weight basis. The immunological activity of octacosapeptide with antisera directed at the Asn^5 to Ser^{13} portion of the molecule, including the ring structure, is somewhat smaller than that of somatostatin on a molar basis. We also observed that with antiserum S 39 (a gift from W. Vale) directed at the NH_2 terminus of somatostatin, the octacosapeptide lacks immunoreactivity. However, because of small amounts of natural material available, a detailed biological and immunological characterization can be made only with synthetic octacosapeptide. The availability of the synthetic octacosapeptide will permit a complete investigation of its biological activities—i.e., the inhibition of the release of GH, thyrotropin, prolactin, insulin, glucagon, gastrin, secretin, cholecystokinin, and other hormones—as well as its exocrine activity, such as inhibition of gastric acid, pepsin, and pancreatic bicarbonate, and other functions.

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