Processing of an anglerfish somatostatin precursor to a hydroxylysine-containing somatostatin 28

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ABSTRACT A novel 28-residue somatostatin (SS) has been isolated from anglerfish pancreatic islets and characterized by complete Edman degradation, peptide mapping, and amino acid analysis. The primary structure of this anglerfish SS-28 (aSS-28) containing hydroxylysine (Hyl) was established to be H-Ser-Val-Asp-Ser-Thr-Asn-Asn-Leu-Pro-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Phe-Tyr-Trp-Hyl-Gly-Phe-Thr-Ser-Cys-OH. This sequence (with the exception of hydroxylysine-23, which is replaced by lysine) is identical to the sequence of the COOH-terminal 28 residues of prepro-SS II predicted on the basis of cDNA analysis [Hobart, P., Crawford, R., Shen, L., Picket, R. & Rutter, W. J. (1980) Nature (London) 288, 137-141]. This is the first instance in which hydroxylysine (to date characteristically observed in collagen or collagen-like structures) has been found in a potential regulatory peptide. Chromatographic characterization of peptides, radiolabeled in islet culture, revealed that aSS-28 contained 80-90% of the radioactivity incorporated into the 8000- to 10000-dalton SS-like polypeptides, whereas 80-90% of this radioactivity was detected in anglerfish SS-14. It appears probable that aSS-28 represents the predominant primary cleavage product derived from prepro-SS II by cleavage at the COOH-terminal side of a single arginine. Based on knowledge of the collagen biosynthesis, it is speculated that hydroxylation may take place as an early post-translational event.

Somatostatin-14 (SS-14), a regulatory peptide discovered on the basis of its inhibition of the secretion of hypophysial growth hormone (1), was characterized by sequence analysis after isolation first from ovine hypothalamus (2, 3) and subsequently from porcine hypothalamus (4), pigeon (5), anglerfish (6), and channel catfish (7) pancreas. Thus, the primary structure of SS-14 was found to be completely conserved in different species and tissues.

It was observed that synthetic SS-14 not only inhibited the secretion of growth hormone with high potency but also the secretion of many other hormones (see ref. 8 for a review), including insulin and glucagon (9).

Based on immunologic and chromatographic evidence (8), multiple forms of SS were recognized to occur besides SS-14 in various tissues of different species. One of these forms, porcine intestinal SS-28 (pSS-28) (10), also identified in extracts of porcine (11) and ovine (12, 13) hypothalamus, was determined by sequence analysis (10-13) to contain the structure of ovine hypothalamic SS-14 (oSS-14) at its COOH terminus. Another form, catfish pancreatic SS-22 (cSS-22), was found by sequence analysis (14, 15) to be significantly different from SS-14 and pSS-28.

Besides the forms of SS mentioned above, 12,000- to 14,000-dalton SS-like polypeptides have been observed (see ref. 16 for a review) and considered as precursors to the smaller forms.

For the investigation of SS precursor processing, the pancreatic islet (Brockmann body) of anglerfish (Lophius americanus) has been especially attractive because of its low content of nonspecific proteases (17) and its relatively high content of SS-containing D cells (18).

We have demonstrated earlier in pulse-chase experiments (17) and by chemical characterization (6) that SS-14 cleaved from 12,000- to 14,000-dalton polypeptide precursors represents the predominant small SS produced by anglerfish pancreatic islets.

By cloning and sequence analysis of cDNA generated from anglerfish pancreatic islet mRNA, the amino acid sequences of two distinct SS precursors were predicted (19). These precursors, prepro-SS I and II, contained at their COOH termini the sequences of oSS-14 or [Tyr7, Gly10]oSS-14, respectively. A third precursor was predicted (20, 21) to differ from prepro-SS I by only two amino acids in the central part of the sequence. SS precursors corresponding to anglerfish prepro-SS I also have been predicted from mammalian (21-23) and catfish (24) mRNAs.

In peptide mapping experiments, we demonstrated (25) that the primary cleavage product of Pro-SS II is not [Tyr7, Gly10]SS-14. We present here the complete chemical characterization of the main primary cleavage product, a 28-residue SS-like polypeptide containing hydroxylated [Tyr7, Gly10]-oSS-14.

EXPERIMENTAL PROCEDURES

Extraction and Purification of aSS-28 After Radiolabeling. After precubation for 30 min at 20°C in Krebs--Ringer bicarbonate buffer, islets were incubated for 5 hr in the presence of pairs of amino acids labeled with 3H and with either 14C or 35S as described (17). After radiolabeling, approximately 110 mg (wet weight) of pancreatic islet tissue was homogenized in 2 M acetic acid with a Potter--Elvehjem homogenizer (300 rpm, 10 strokes). After centrifugation (800 × g for 10 min at 25°C), the supernatant was desalted with a Bio-Gel P-2 (100--200 mesh) column (2.5 × 18 cm) equilibrated with 2 M acetic acid. The material (2.24 mg of protein), which was eluted in the void volume, was subjected to gel filtration through a Bio-Gel P-30 column as described in Fig. 1. Approximately 125 μg of protein was eluted with partition coefficients of 0.3-0.5, corresponding to a molecular weight range of 8000-2500, concentrated by lyophilization, dissolved in 3 M acetic acid, and filtered through 0.22-μm filters. The filtered peptides were

Abbreviations: SS, somatostatin; oSS-14, ovine hypothalamic SS-14; aSS-28, anglerfish pancreatic islet SS-28; pSS-28, porcine intestinal SS-28; cSS-22, catfish pancreatic SS-22; > PhNCs, phenylthiohydantoin; PhNHCS, phenylthiocarbamyl; RP-HPLC, reversed-phase HPLC; CM, carboxymethylated.
usually S-carboxymethylated (13) and subsequently purified in two steps with reversed-phase HPLC (RP-HPLC) using Vydac C18 columns (0.46 × 25 cm; particle size, 5 μm; pore size, 330 Å) and an aqueous mixture of 0.1% trifluoroacetic acid and acetonitrile for elution. Extraction and purification were carried out at 23°C and monitored by protein determination as described by Bradford (26).

**Amino Acid Analysis.** Peptides (0.3–5.0 μg) were hydrolyzed for 22–24 hr at 110°C with 25 μl of 4 M methanesulfonic acid, 0.2% tryptamine, or 2 M NaOH in the presence of 1 nmol of norleucine as internal standard. The hydrolysates were treated with 25 μl of 3.5 M NaOH or 2.3 M methanesulfonic acid plus 0.12% tryptamine, respectively, and applied to a Beckman 121 MB amino acid analyzer using sodium or lithium citrate buffers for elution and ninhydrin for detection. The coefficient of variation was usually between 1% and 10%. More details are published elsewhere (13).

**Edman Degradation.** Peptides (0.5–2.0 nmol) were degraded in the presence of 3–4 mg of Polybrene in an automatic spinning cup sequencer (27) with a single-coupling single-cleavage program as described (28, 29). Phenylthiohydantoin (> PhNCS)-conjugated amino acids were determined with RP-HPLC (coefficient of variation, 1–6%; minimal detectable amount, <10 pmol) (28). The main > PhNCS derivative of 5-hydroxylysine (Calbiochem) subjected to automatic Edman degradation and RP-HPLC was presumably > PhNCS-N′-phenylthiocarbamyl (PhNHCNS)-5-hydroxylysine, which was eluted between > PhNCS-dehydrothreonine and > PhNCS-methionine with an absorption maximum at 262 nm. Back hydrolysis of the collected > PhNCS derivative of 5-hydroxylysine with constant boiling HCl containing 0.1% SnCl2 (30), 3 μl of thioglycol/ml, and norleucine as internal standard (for 15–27 hr at 140°C) yielded a product that could not be distinguished from 5-hydroxylysine with the amino acid analyzer using sodium or lithium citrate buffers.

**RESULTS**

As mentioned above, we had established (25) that [Tyr7, Gly10]SS-14, which was predicted (19) to represent the COOH-terminal sequence of prepro-SS II, was not expressed as such. Therefore, we screened pancreatic extracts (after tissue culture in the presence of various radioactive amino acids) for 8000- to 10000-dalton polypeptides containing most of the amino acids constituting [Tyr7, Gly10]SS-14. This screening was carried out by gel filtration of the extracted polypeptides and subsequent RP-HPLC of pools of the 8000- to 2500-dalton and 2500- to 10000-dalton polypeptides. In the pool of the 2500- to 10000-dalton polypeptides, we did not find a species that was labeled with radioactive tyrosine, glycine, cysteine, and tryptophan as expected for an extended sequence of [Tyr7, Gly10]SS-14. However, the pool of the 8000- to 25000-dalton polypeptides contained a species that could be radiolabeled with tyrosine, glycine, tryptophan, cysteine, asparagine, threonine, valine, arginine, lysine, and leucine. Chromatographic results of one labeling experiment in which the pair [3H]tryptophan and [14C]lysine was used is presented as an example for this approach (Fig. 1).

In subsequent experiments with RP-HPLC, it was revealed that the species discovered in the pool of the 8000- to 2500-dalton polypeptides was mainly composed of two components, which later were identified as the 28-residue SS-like polypeptides aSS-28 and [Lys37]aSS-28. aSS-28, which eluted before [Lys37]aSS-28, represented the predominant form, carrying >95% of the total radioactivity of the 28-residue SS-like polypeptides. Analysis of the distribution of radioactivity over the 8000- to 10000-dalton SS-like polypeptides showed that ~88–90% of the radioactivity found in SS-like peptides was incorporated in SS-14, whereas 12–10%

**FIG. 1.** Gel filtration of radiolabeled islet peptides. Approximately 105 mg (wet weight) of freshly dissected islet tissue was incubated for 5 hr at 20°C with [3H]tryptophan (4.5 Ci/mmol; 1 Ci = 37 GBq) and [14C]lysine (345 mCi/mmol). The tissue was subsequently washed, extracted, and applied to a (1.6 × 95 cm) column packed with Bio-Gel P-30 (100–200 mesh) and equilibrated with 2 M acetic acid. Arrows mark the void volume and the elution volumes of anglerfish proinsulin (aP-I), insulin (al), glucagon (aG)/aSS-28 (and [Lys37]aSS-28), and SS-14. The bar indicates the pool of the 8000- to 2500-dalton polypeptides subjected to RP-HPLC for further purification.

The data derived from Edman degradation were confirmed and supplemented by peptide mapping of CM-aSS-28 fragments obtained by digestion with thermolysin (Fig. 3). Based on the absorbance profile (Fig. 3) and amino acid analysis (Table 1), the major fragments cleaved from natural CM-aSS-28 were identified to be CM-aSS-28-(1–19)-OH, aSS-28-(1–24)-OH and CM-aSS-28-(25–28)-OH. The fragment pattern matched with the substrate specificity of

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thermolysin, which has been observed to cleave preferentially peptide bonds at the NH$_2$-terminal side of hydrophobic amino acids as long as they are not COOH-terminally linked to proline (31). The retention time of CM-aSS-28-(25-28)-OH did not deviate significantly from the retention time of CM-oSS-14-(11-14)-OH cleaved from synthetic CM-oSS-14 with thermolysin. However, aSS-28-(25-28)-OH was eluted significantly later than its COOH-terminally amidated analog CM-oSS-14-(11-14)-NH$_2$ cleaved from synthetic CM-oSS-14-NH$_2$. Therefore, it was concluded that CM-aSS-28, and thus aSS-28, carry a free COOH-terminal carboxylic group.

aSS-28-(20-24)-OH containing hydroxylysine-23 was resolved from its nonhydroxylated analog [Tyr$^2$, Gly$^{10}$]-oSS-14-(6-10)-OH cleaved from synthetic [Tyr$^2$, Gly$^{10}$]-oSS-14.

The purity of natural CM-aSS-28 was estimated to be >90% on the basis of RP-HPLC (Fig. 2), deviation of amino acid ratios from integers in amino acid analysis (Table 1), Edman degradation, and peptide mapping on RP-HPLC (Fig. 3).

Since hydroxylysine of collagen has often been found to be linked O-glycosidically to glucose or galactose or glucosylgalactose (32), the possibility that hydroxylysine-23 of CM-aSS-28 might be glycosylated was examined. We followed closely a procedure that had been used by Brownell and Veis (33) in the characterization of carbohydrate derivatives of hydroxylysine of collagen. This procedure is based on the higher stability of acetal bonds under alkaline conditions compared to acidic conditions. When purified human collagen, type II, was hydrolyzed with 4 M methanesulfonic acid and 2 M NaOH, the amino acid ratios of hydroxylysine to the sum of hydroxylysine and lysine were 1/2.2 and 1/3.3, respectively. The finding of a lower ratio after alkaline hydrolysis indicated the presence of carbohydrate linked to hydroxylysine. For CM-aSS-28, a ratio of 1/3.1 was independent of the type of hydrolysis applied. Thus, it was concluded that hydroxylysine-23 of CM-aSS-28 probably was not glycosylated. Partial glycosylation appeared highly improbable in view of the peptide mapping experiment (Fig. 3), which provided evidence for only one hydroxylysine-containing fragment.

On the basis of the data presented here, Edman degradation, peptide mapping, and amino acid analysis, the primary structure of aSS-28 was established (see Fig. 4). With a similar approach, the minor 28-residue SS-like polypeptide of pancreatic islets was completely characterized to be [Lys$^{21}$]-aSS-28 (37).

DISCUSSION

In the process of screening for an SS-like polypeptide containing the sequence of [Tyr$^2$, Gly$^{10}$]-oSS-14, we discovered and completely characterized a novel somatostatin, aSS-28, the sequence of which corresponds to the COOH-terminal 28 residues of prepro-SS II except for lysine-23, which is replaced by hydroxylysine.

The finding of hydroxylysine in aSS-28 is surprising because to date this amino acid has only been observed as a component of collagen and collagen-like structures (32). In collagen, hydroxylysine is located NH$_2$-terminally to glycine...
residues. It has been shown that collagen hydroxylysine provides sites for glycosylation and crosslinking (32). For the latter reaction, oxidative desamination of the hydroxylysine side chain seems to be required. Since hydroxylysine-23 of aSS-28 was neither observed to be glycosylated nor desaminated, its presence may be suggestive for a spectrum of (pancreatic) biologic activities of aSS-28 significantly different from the spectrum of SS-14 activities.

This view is supported also by a structural comparison of aSS-28 with other peptides of the somatostatin family (Fig. 4). Because of the significant size variation within this peptide family, the sequences of the smaller peptides have to be compared with the corresponding COOH-terminal parts of the larger peptides. Thus, the sequence homologies of aSS-28 with [Lys\(^{23}\)]aSS-28, pSS-28 (10-13), oSS-14 (2-7), and cSS-22 (14, 15) were calculated to be 96%, 61%, 79%, and 23%, respectively. A sequence comparison between aSS-28 and oSS-14 reveals that the primary sequence of aSS-28 deviates significantly from the sequence of oSS-14 in the central part, oSS-14 (6-11) which (with the exception of threonine-10) has been recognized (34) to be essential for the potent inhibition of growth hormone secretion from cultured rat anterior pituitary cells. Thus, aSS-28 is not expected to be a potent inhibitor of hypophyseal growth hormone secretion, as has been shown already for the synthetic analog [Tyr\(^7\), Gly\(^{10}\)]oSS-14 (Wylie Vale, personal communication).

It has been demonstrated in experiments with free and membrane-bound ribosomes (35) that hydroxylation of lysine residues in collagen is apparently catalyzed by membrane-bound lysyl hydroxylase and probably represents a cotranslational event. A similar mechanism may hold for the hydroxylation of aSS-28. In view of the location of the hydroxylation site in the COOH-terminal sequence of aSS-28, and in view of the size of the participating subcellular structures such as ribosomes and membranes, hydroxylation may take place in this instance as an early post-translational event (Fig. 5).

Based on the pattern of the radioactivity distribution in the

![Fig. 3. Peptide mapping of natural CM-aSS-28 on RP-HPLC. CM-aSS-28 (≈ 2.0 nmol) was digested in 50 μl of 0.2 M N-ethylmorpholine (adjusted to pH 8.0 with trifluoroacetic acid)/2 mM CaCl\(_2\)/5% (vol/vol) 1-propanol containing thermolysin (substrate-to-enzyme weight ratio, 20:1) for 1 hr at 30°C. Subsequently, the mixture was applied to RP-HPLC with a Perkin–Elmer series 4 liquid chromatograph equipped with a Kratos Spectroflow 773 monitor. Chromatography was carried out on a Vydac C\(_18\) column as described in the legend to Fig. 2. All products detected in the eluate on the basis of their absorbance were subjected to amino acid analysis. Products I–III contained <0.1 μg of peptide. The identity of major products [CM-aSS-28(25-28)-OH, 1.0 μg; CM-aSS-28(1-19)-OH, 2.0 μg; aSS-28-(20-24)-OH, 1.2 μg] is indicated (see also Table 1). Products IV (0.26 μg of peptide) and V (0.13 μg) probably represent aSS-28-(21-24)-OH and CM-aSS-28-(1-20)-OH, respectively. The retention times of CM-oSS-14(11-14)-NH\(_2\) (FTSC\(^\circ\)) and [Tyr\(^7\), Gly\(^{10}\)]oSS-14(6-10)-OH (FYWKG) are also denoted by arrows. The fragmentation is illustrated at the top of the figure with major (I) and minor (II) cleavage sites. A, alanine; C, cysteine (all cysteine residues shown here were carboxymethylated); D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; K*\(^\circ\), hydroxylysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; \(\circ\), amide.](image-url)
gel filtration and RP-HPLC of radiolabeled peptide fragments, it has been concluded that aSS-28 represents the predominant smallest SS-like polypeptide containing the (modified) sequence of [Tyr^{1}, Gly^{19}]SS-14 derived from prepro-SS II. In view of the appearance of aSS-28 in the early chase period of pulse–chase experiments (unpublished results), it seems probable that aSS-28 represents the predominant primary cleavage product in the processing of anglerfish prepro-SS II (Fig. 5).

It is interesting that anglerfish prepro-SS I is processed to SS-14 by proteolytic cleavage at the COOH-terminal side of a pair of basic residues (-Arg-Lys-), whereas prepro-SS II, which also contains these residues and is structurally homologous in the environment of this dibasic pair, was not observed to be cleaved at this site. Instead cleavage was found to occur between a single arginine and serine-1 of aSS-28. An equivalent cleavage of an SS-28 from anglerfish prepro-SS I has not been detected. In contrast with these findings, both mammalian SS-28 and SS-14 may be cleaved from the mammalian SS precursor corresponding to anglerfish prepro-SS I (Fig. 5). To date, a mammalian equivalent of prepro-SS II has not been discovered.

The observation of different processing pathways is suggestive for either a high substrate specificity of the proteases involved in the SS precursor conversion (36) or the localization of precursors or proteases in different cells, or both.

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