Characterization of three peptides derived from prosomatostatin [prosomatostatin-(1–63)-, -(65–76)- and -(79–92)-peptides] in a human pancreatic tumour

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By using only reverse-phase h.p.l.c., three fragments of prosomatostatin were isolated from an extract of a human pancreatic neuroendocrine tumour that produced somatostatin, vasoactive intestinal polypeptide and gastrin-releasing peptide. The amino acid composition of the peptides indicated that they represented prosomatostatin-(1–63)-peptide, prosomatostatin-(65–76)-peptide and prosomatostatin-(79–92)-peptide (somatostatin-14). The identity of prosomatostatin-(1–63)-peptide was confirmed by characterization of the products of digestion with *Armillaria mellea* (honey fungus) proteinase. Partial micro-sequencing of prosomatostatin-(1–63)-peptide showed that the Gly²⁴–Ala²⁵ bond of preprosomatostatin was the site of cleavage of the signal peptide. Thus human prosomatostatin is a protein of 92 amino acid residues that is proteolytically cleaved in a pancreatic tumour at the site of a dibasic-residue (arginine–lysine) processing site and at a single-monobasic-residue (arginine) processing site.

INTRODUCTION

The production of somatostatin by neuroendocrine tumours in diverse locations has been extensively documented [reviewed in Vehmeyer *et al.* (1985)]. Tumours in which somatostatin-producing D-cells represent a majority cell type ('somatostatinomas') are rare, but have been found most often in the pancreas (Ganda *et al.*, 1977; Larsson *et al.*, 1977; Krejs *et al.*, 1979; Pipeleers *et al.*, 1979; Wright *et al.*, 1980). The structure of the biosynthetic precursor of somatostatin (preprosomatostatin) in the human may be deduced from the nucleotide sequence of a cloned cDNA prepared from a human pancreatic tumour (Shen *et al.*, 1982), and the structure of the gene has been determined from DNA fragments from a human genomic library (Shen & Rutter, 1984). Human preprosomatostatin is a protein of 116 amino acids that contains the sequence of somatostatin-14 at its C-terminus. In the present investigation, isolation and characterization of peptides representing fragments of prosomatostatin from a pancreatic neuroendocrine tumour has enabled the elucidation of the pathway of post-translational processing of the prohormone.

EXPERIMENTAL

Patient details

The patient was a 61-year-old man with a 2-year history of abdominal pain, watery diarrhoea and flushing after exercise. Exploratory laparotomy revealed a tumour (4 cm × 3 cm) in the caudal and corpus regions of the pancreas with numerous metastases in regional lymph nodes and the liver. The fasting plasma concentration of vasoactive intestinal polypeptide was 190 pmol/l (healthy subjects < 2 pmol/l) and the fasting

plasma somatostatin level was 367 pmol/l (healthy subjects < 20 pmol/l). After laparotomy, the patient developed a fulminant watery diarrhoea hypokalaemia achlorohydrria syndrome (Verner & Morrison, 1974). In April 1986, the primary tumour was resected, but this reduction in tumour mass did not improve the patient's condition. Subsequent embolization of the hepatic artery resulted in normalization of the patient's diarrhoea and a reduction in the severity of the flush. Treatment with human leucocyte interferon was initiated, and the plasma vasoactive-intestinal-polypeptide levels normalized and the flush disappeared temporarily. Another embolization of the liver was required in December 1986.

Tumour histopathology

Tumour tissue was fixed in neutral-buffered formalin and embedded in paraffin. Immunocytochemistry was performed using the peroxidase–anti-peroxidase technique (Sternberger, 1979) using monoclonal antisera against 5-hydroxytryptamine (Consolazione *et al.*, 1981) and chromogranin A (Lloyd & Wilson, 1983) and polyclonal antisera against other regulatory peptides (El-Salhy, 1984). The primary pancreatic tumour was non-encapsulated, and the tumour cells showed various extents of polymorphism, but low mitotic activity. The tumour showed several growth patterns (solid, trabecular and nodular) in different regions. The stromal component was also variable. The surrounding pancreas showed a variable extent of acinar atrophy and a chronic inflammatory reaction. The islets of Langerhans were enlarged, with a normal distribution of insulin, glucagon and somatostatin cells, but few pancreatic polypeptide cells.

Almost all tumour cells showed an argyrophil (Grimelius) reaction, indicating the presence of neuro-

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hormonal secretory granules, but no argentaffin (Masson) reaction was observed. Most tumour cells displayed chromogranin A and neuron-specific enolase immunoreactivity, and a smaller proportion of cells showed vasoactive-intestinal-polypeptide, somatostatin and insulin immunoreactivity. No pancreatic polypeptide, gastrin, neurotensin, 5-hydroxytryptamine or S-100-protein immunoreactivities were observed.

Extraction of tumour tissue

Tissue from the primary tumour (0.71 g) was placed in liquid N₂ immediately after resection and stored at -70 °C until time of extraction. The tissue was homogenized while still frozen with ethanol/0.7 M-HCl (3:1, v/v; 20 ml) with an Ultra-Turrax blender. The homogenate was stirred at 4 °C for 16 h, centrifuged (1600 g for 1 h at 4 °C) and ethanol removed from the supernatant under reduced pressure. Peptides were isolated from the extract by using Sep-pak C₁₈ cartridges (Waters Associates, Milford, MA, U.S.A.) (Conlon & McCarthy, 1984). After centrifugation (20000 g for 1 h at 4 °C), the supernatant was pumped at a flow rate of 1 ml/min through three Sep-pak cartridges connected in series. Bound material was eluted with 70 % (v/v) acetonitrile and freeze-dried.

Purification of the peptides

The tumour extract, after partial purification on Sep-pak cartridges, was redissolved in 0.1 % (v/v) trifluoroacetic acid (2 ml) and injected on to a Vydac 218TP54 reverse-phase C₁₈ h.p.l.c. column (1 cm × 25 cm) (Separations Group, Hesperia, CA, U.S.A.) equilibrated at 30 °C and at a flow rate of 1.5 ml/min with 0.1 % trifluoroacetic acid. The concentration of acetonitrile in the eluting solvent was raised to 21 % (v/v) over 10 min and to 49 % (v/v) over 50 min. Absorbance was measured at 280 nm and 214 nm, and prominent peaks were collected manually. Prosomatostatin-(1-63)-peptide was purified to homogeneity on a Supelcosil LC-3DP phenyl column (0.46 cm × 25 cm) (Supelco, Bellefonte, PA, U.S.A.) eluted at 30 °C at a flow rate of 1.5 ml/min with a linear gradient (75 ml) formed from acetonitrile/water/trifluoroacetic acid (280:719:1, by vol.) and acetonitrile/water/trifluoroacetic acid (420:579:1, by vol.).

Digestion with *Armillaria mellea* (honey fungus) proteinase

Prosomatostatin-(1-63)-peptide (approx. 3 nmol) was incubated at 30 °C for 16 h with *Armillaria mellea* proteinase (Lewis *et al.*, 1978) (60 pmol) in 50 mM-*N*-ethylmorpholine buffer, pH 8.0 (200 µl). The reaction mixture was injected on to a Supelcosil LC-18-DB column (1 cm × 25 cm) equilibrated with 0.1 % (v/v) trifluoroacetic acid at 30 °C and eluted at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 49 % (v/v) over 60 min.

Structural analysis

Amino acid compositions were determined by using a Durrum automatic analyser (Conlon & McCarthy, 1984). Hydrolysis was carried out by using approx. 2 nmol of peptide in evacuated tubes containing 5.7 M-HCl (200 µl) and phenol [20 µl of a 2.5 % (w/v) solution]. Incubations were for 24 h at 110 °C. Cysteine and tryptophan residues were not measured. Amino acid

sequence analysis was carried out by automated Edman degradation using an Applied Biosystems model 470A gas-phase sequencer (Thim *et al.* 1987). The detection limit for amino acid phenylthiohydantoin derivatives was 0.5 pmol.

Radioimmunoassay methods

Somatostatin was measured using an antiserum directed against the central residues of somatostatin-14 that shows full reactivity with somatostatin-28 (McIntosh *et al.*, 1978). Vasoactive intestinal polypeptide was measured using an antiserum directed against the *N*-terminal-to-central residues of the porcine peptide (Agoston *et al.*, 1985). Gastrin-releasing peptide was measured by using an antiserum directed against the *C*-terminal region of the porcine peptide that shows full reactivity with neuromedin C (Conlon *et al.*, 1986). All concentrations are expressed relative to synthetic standards from Peninsula Laboratories (Europe) Ltd. (St. Helens, Lancs., U.K.).

RESULTS

Concentrations of regulatory peptides in the tumour extract

The extract of primary tumour tissue contained 49.9 nmol equivalents of somatostatin/g wet weight, 29 pmol equivalents of vasoactive intestinal polypeptide/g and 17 pmol equivalents of gastrin-releasing peptide/g. Immunoreactive substance P and neurokinin A were not detected in the extract. The detection limits for substance P and neurokinin A are 3 fmol/tube and 1 fmol/tube respectively.

Purification of the prosomatostatin-derived peptides

The elution profile on reverse-phase h.p.l.c. of the tumour extract, after concentration on Sep-pak cartridges, is shown in Fig. 1. The retention times of peaks 1 and 2 suggested possible identity with somatostatin-28-(1-12)-peptide [prosomatostatin-(65-76)-peptide] and somatostatin-14. Peak 2 was the only peak in the chromatogram with somatostatin-like immunoreactivity determined by radioimmunoassay. Peak 3 [prosomatostatin-(1-63)-peptide] was purified to apparent homogeneity on a diphenylmethylsilyl-silica column (Fig. 2). The approximate final yields of the purified peptides (per 0.71 g of tissue) were: prosomatostatin-(1-63)-peptide, 8 nmol; prosomatostatin-(65-76)-peptide, 12 nmol; and somatostatin-14, 24 nmol. Yields were determined by amino acid analysis, but should be regarded as approximate, since efficiencies of extraction and recoveries during chromatography are probably different for the three peptides.

Characterization of the prosomatostatin-derived peptides

The amino acid compositions of the purified peptides from peaks 1 and 2 (Fig. 1) and peak 3 (Fig. 2) are shown in Table 1. The data confirm that peak 1 is prosomatostatin-(65-76)-peptide and peak 2 is somatostatin-14. The composition of the peptide from peak 3 indicated probable identity with prosomatostatin-(1-63)-peptide. With the exception of a slightly high value for glutamic acid/glutamine and a slightly low value for proline, the agreement between the composition measured and that predicted from the nucleotide sequence of the prepro-

Fig. 1. Reverse-phase h.p.l.c. on a Vydac 218TP column of an extract of a somatostatin-producing pancreatic tumour after partial purification on Sep-pak cartridges

Details of the elution conditions are given in the text. The broken line shows the concentration of acetonitrile in the eluting solvent. The arrows show the retention times of somatostatin-28-(1-12)-peptide (SS-28₁₋₁₂), somatostatin-28 (SS-28) and somatostatin-14. (SS-14) Peaks *1-*3 were purified further.

Fig. 2. Purification of prosomatostatin-(1-63)-peptide on a reverse-phase Supelcosil LC-3DP phenyl h.p.l.c.

The concentration of acetonitrile in the eluting solvent is shown by the broken lines and prosomatostatin-(1-63)-peptide is denoted by the asterisk.

somatostatin gene (Shen *et al.*, 1982; Shen & Rutter, 1984) was good. Cleavage of prosomatostatin-(1-63)-peptide with *Armillaria mellea* proteinase generated five peptide fragments, which were separated by reverse-phase h.p.l.c. (Fig. 3). The amino acid compositions of the peptides (Table 2) indicated that they were prosomatostatin-(1-7)-peptide, prosomatostatin-(8-12)-peptide, prosomatostatin-(13-20)-peptide, prosomatostatin-(21-25)-peptide and prosomatostatin-(26-63)-peptide. Formation of these fragments resulted from the expected cleavage of the peptide bond at the *N*-terminal side of the three lysine residues in the substrate and also from cleavage of the Leu⁷-Arg⁸ bond. Prosomatostatin-(1-63)-

Table 1. Amino acid compositions of peptides derived from prosomatostatin isolated from a human pancreatic tumour

Results are the means of two determinations. Values in parentheses are those predicted from the nucleotide sequence of the preprosomatostatin gene.

Amino acid	Prosomato-statin peptide ...	Relative amount		
		(1-63)	(65-78)	(79-92)
Asx		5.7 (6)	1.9 (2)	1.1 (1)
Thr		1.0 (1)		2.0 (2)
Ser		4.1 (4)	2.2 (2)	1.0 (1)
Glx		17.1 (16)	1.4 (1)	
Pro		3.4 (4)	2.1 (2)	
Gly		1.3 (1)		1.1 (1)
Ala		10.0 (10)	2.9 (3)	1.0 (1)
Leu		11.4 (11)		
Met		0.9 (1)	0.7 (1)	
Tyr		1.1 (1)		
Phe		2.1 (2)		3.1 (3)
Lys		3.2 (3)		2.0 (2)
Arg		3.1 (3)	1.2 (1)	

peptide was subjected to four cycles of automated Edman degradation (Table 3). The *N*-terminal sequence of the peptide (Ala-Pro-Ser-Asp) corresponds to residues 25-28 of preprosomatostatin. Thus the Gly²⁴-Ala²⁵ bond is identified as the site of cleavage of the signal peptide.

DISCUSSION

The very high level of expression of the preprosomatostatin gene in a pancreatic neuroendocrine

tumour has permitted the isolation of peptides derived from prosomatostatin that do not contain the somatostatin-14 sequence. Previous studies have identified somatostatin-14 and prosomatostatin-(65-76)-peptide in human tumour tissue (Patel *et al.*, 1983; Böhlen *et al.*, 1983; Conlon & McCarthy, 1984), but the present investigation has led to characterization of the *N*-terminal fragment. The identity of prosomatostatin-(1-63)-peptide was confirmed by determination of the amino acid compositions of the peptide fragments generated by digestion with *Armillaria mellea* proteinase. Although

Fig. 3. Reverse-phase h.p.l.c. on a Supelcosil LC-18-DB column of the reaction mixture after digestion of prosomatostatin-(1-63)-peptide with *Armillaria mellea* proteinase

The concentration of acetonitrile in the eluting solvent is shown by the broken line. The peptide fragments were identified by amino acid composition (Table 2) as: peak 1, KQELA [prosomatostatin-(27-35)-peptide]; peak 2, KSLAAAAG [prosomatostatin-(13-20)-peptide]; peak 3, APSDPRL [prosomatostatin-(1-7)-peptide]; peak 4, RQFLQ [prosomatostatin-(8-12)-peptide]; and peak 5, KYFLAELLSEPNQTENDALEPEDLSQA AEQDEMRLELQ [prosomatostatin-(26-63)-peptide].

the primary specificity of the enzyme is considered to be cleavage at the *N*-terminal side of lysine residues (Lewis *et al.*, 1978), cleavage at the *N*-terminal side of the arginine residue at position 8 in the substrate was observed. In a previous study in which aspartate aminotransferase was digested with the enzyme, cleavage was seen at the site of three arginine residues (Doonan *et al.*, 1975). In this case, however, cleavages were at the *C*-terminal side of arginine. As in the rat (Goodman *et al.*, 1983) and pig (Schmidt *et al.*, 1985), the signal peptide is removed from preprosomatostatin by cleavage at the Gly²⁴-Ala²⁵ bond. Formation of prosomatostatin-(1-63)-peptide requires cleavage at a single arginine residue, and it is probable that the specificity of such cleavages is regulated by conformational features in the prohormone (Schwartz, 1986). Prosomatostatin-(1-63)-peptide contains three arginine residues, but these sites are not recognized by the processing enzyme(s). The formation of prosomatostatin-(1-63)-peptide and prosomatostatin-(65-76)-peptide indicates the Arg⁶⁴ residue is removed by the action of exopeptidase(s).

Previous studies (Conlon *et al.*, 1981; Böhlen *et al.*, 1983) have identified somatostatin-28 in extracts of human pancreatic tumours. The major peaks of u.v.-absorbing material with retention times similar to that of synthetic somatostatin-28 (Fig. 1) were not associated with somatostatin-like immunoreactivity. It is concluded, therefore, that the concentration of this component in the tumour must be less than 1 nmol/g. Similarly, peptides identified as prosomatostatin-(68-76)-peptide and prosomatostatin-(65-74)-peptide were also isolated from a pancreatic tumour (Conlon & McCarthy, 1984). These peptides were eluted from a reverse-phase h.p.l.c. column before prosomatostatin-(65-76)-peptide. Consequently, the elution profile shown in Fig. 1 shows either that these metabolites were not present in the tumour extract or that they did not bind to the Sep-pak cartridges during the initial isolation step. Identification of prosomatostatin-(1-63)-peptide does not preclude the formation of other peptides derived from preprosomatostatin that were not isolated in this study. The lower yield of prosomatostatin-(65-76)-peptide compared with

Table 2. Amino acid composition of peptides derived from prosomatostatin-(1-63)-peptide by digestion with *Armillaria mellea* proteinase

The values in parentheses are those predicted from the nucleotide sequence of the preprosomatostatin gene.

Amino acid	Prosomatostatin peptide...	Relative amount				
		(1-7)	(8-12)	(13-20)	(21-25)	(26-63)
Asx		1.0 (1)				4.8 (5)
Thr						1.0 (1)
Ser		1.0 (1)		1.0 (1)		1.9 (2)
Glx			2.2 (2)		2.0 (2)	12.6 (12)
Pro		2.0 (2)				1.9 (2)
Gly						
Ala		1.0 (1)		3.9 (4)	1.1 (1)	3.9 (4)
Leu		1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	6.8 (7)
Met						0.9 (1)
Tyr						1.0 (1)
Phe			0.9 (1)			1.0 (1)
Lys				1.0 (1)	0.9 (1)	
Arg		1.0 (1)	1.0 (1)			1.0 (1)

Table 3. Determination of the N-terminal sequence of prosomatostatin-(1-63)-peptide by automated Edman degradation

Cycle no.	Amino acid phenylthiohydantoin derivative	Yield (pmol)
1	Ala	529
2	Pro	576
3	Ser	84
4	Asp	239

somatostatin-14 would be consistent with the formation of prosomatostatin-(1-76)-peptide, i.e. the prosomatostatin molecule lacking Arg-Lys-somatostatin-14. This peptide has been isolated from rat brain (Benoit *et al.*, 1984). On the other hand, the hydrophilic prosomatostatin-(65-76)-peptide does not bind efficiently to the Sep-pak cartridges used in the isolation procedure, so that manipulative losses may be responsible for the low yield. Somatostatin-14 was the only major component in the tumour extract with somatostatin-like immunoreactivity, demonstrating that intact prosomatostatin (residues 1-92) was either absent or present in very low concentrations. Previous studies have demonstrated that prosomatostatin in rat brain (Benoit *et al.*, 1984) and pig intestine (Schmidt *et al.*, 1985) is cleaved at the Leu³²-Leu³³ bond. The extraction methods used in these studies involved, however, boiling in aqueous solution, and it is possible that this procedure resulted in proteolysis at an unphysiological site. In contrast, the tumour tissue was extracted at low temperature with a 75% (v/v) ethanolic solution, so that 'artefactual' activation of a protease under these condition is unlikely.

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