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CHARACTERIZATION OF THE CARBOXYL TERMINAL FLANKING PEPTIDE OF RAT PROGASTRIN

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SUMMARY: A peptide identical in structure to the carboxyl-terminal flanking nonapeptide of rat progastrin, predicted by cDNA sequence, was synthesized. The synthetic peptide was used for production of a rabbit antiserum. This antiserum was used to develop a radioimmunoassay specific for rat carboxyl terminal flanking peptide. This assay was used to monitor the purification of immunoreactivity from rat antral extracts. Gel permeation, anion exchange and reverse phase chromatography steps resulted in a single absorbance peak associated with the carboxyl terminal flanking peptide immunoreactivity. The purified peptide eluted in the same position as the synthetic peptide during all three types of chromatography. This material was shown to be identical in mass to Ser-Ala-Glu-Glu-Glu-Asp-Gln-Tyr-Asn, the predicted sequence of the carboxyl terminal nonapeptide of rat progastrin.

The chemical structure of gastrin was one of the first of the gastrointestinal peptides to be determined (1). Based on the structure of gastrin, oligonucleotide probes were made that led to cloning and determination of the complete structure of gastrin cDNA from pig (2), human (3,4) and rat (5). The only portion of processed rat progastrin that has been chemically determined is that of the heptadecapeptide (little gastrin) (6).

Investigators have developed region specific radioimmunoassays based on structures predicted from cDNA sequences for various portions of progastrin. Dockray has developed specific radioimmunoassays for the carboxyl terminal region of porcine and human progastrin. These assays have been used to study the processing of progastrin in these species (7,8). Post-translational processing of porcine progastrin includes phosphorylation of a serine residue in the COOH terminal fragment (7). In human gastrinoma tissue, larger COOH terminal immunoreactive peptides were detected than in normal antral tissue (8). We have synthesized the carboxyl terminal portion of rat progastrin, developed a specific radioimmunoassay, and used the radioimmunoassay to monitor the purification of immunoreactivity from rat antral extracts. The purified material was characterized by elution positions during gel permeation, anion exchange, and reverse phase chromatographies and by mass spectral analysis.

METHODS

Synthesis of COOH-terminus of rat progastrin. The carboxyl terminal flanking peptide (CTFP) of rat progastrin, Ser-Ala-Glu-Glu-Glu-Asp-Gln-Tyr-Asn (96-104 rat preprogastrin), which corresponded to

Abbreviations: CTFP, carboxyl terminal flanking peptide.

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the carboxyl terminus of rat progastrin was synthesized on a Biosearch Sam II synthesizer, using the Merrifield solid phase technique (9) and the manufacturer's recommended conditions. The peptide was cleaved from the resin by HF and the peptide was purified by reverse phase chromatography on a 20mm x 25 cm Vydac C-18 column. The quality of the purified peptide was evaluated by amino acid analysis, mass spectral analysis and by isocratic HPLC elution profile.

Amino acid analysis of synthetic CTFP. Approximately 500 pmoles of the purified synthetic peptide were hydrolyzed in 6 M HCl for 22 h at 110^{o}C. The hydrolysate was dried, dissolved in dilution buffer and applied to a Beckman 6300 amino acid analyzer. The eluted amino acids were derivatized with ninhydrin. The derivatives were detected by absorbance at 440 and 540 nm and quantitated on a Nelson Analytical System.

Radioimmunoassay development. The CTFP (96-104 rat preprogastrin) was coupled to keyhole limpet haemocyanin with N-Ethyl-N(2-dimethyl)-aminopropyl-carbodiimide. Eight week old rabbits were immunized with multiple intradermal injections of emulsions of conjugated antigen and complete Freund's adjuvant. The peptide was labeled by the chloramine T method (10) and the label purified by Sephadex G-10 chromatography followed by AE41 anion exchange chromatography. After two booster injections the ID_{50} of antiserum 8771 (used in these studies) was 20 fmol/ml. This antiserum had less than 0.1 per cent crossreactivity with gastrin or glycine extended gastrin.

Extraction of rat antra. Freely fed male Sprague-Dawley rats were killed by decapitation and the antral portion of the stomach quickly removed and rinsed. The rinsed antra were extracted with boiling water (10 ml/g tissue) containing protease inhibitors EDTA, PMSF, pepstatin A and leupeptin. After boiling for 10 min the antra were homogenized and the extracts centrifuged for 30 min at 20,000 x g.

Analytical gel permeation chromatography. The antral extract supernatant (2 ml) was loaded onto a Sephadex G-50 SF (1 x 95 cm) gel permeation column equilibrated in 0.05 M sodium barbital pH 8.4 containing 0.1% bovine serum albumin. Radiolabeled albumin was used to mark the void volume and $Na^{125}I$ was used to calibrate the total column volume. The column was eluted with equilibration buffer and 1 ml fractions were analyzed for rat CTFP immunoreactivity.

Purification of rat CTFP immunoreactivity. The antral extract supernatant (40 ml) was loaded onto a Sephadex G-50SF column (5 cm x 90 cm) equilibrated in 0.1% ammonium bicarbonate. The column was eluted with the same buffer and 10 ml fractions were analyzed for rat CTFP immunoreactivity. The latest eluting peak was pooled and diluted to the conductivity of 0.1M Tris-HCl with water. The CTFP immunoreactivity was divided into three equal portions and loaded onto a Pharmacia Mono Q FPLC column equilibrated in 0.1 M Tris-HCl pH 8.3. The column was eluted with a linear gradient to 0.5 M NaCl. Fractions (1 ml) were assayed for CTFP immunoreactivity. The major peak of CTFP immunoreactivity was diluted (1:3) with 0.1 % trifluoroacetate and loaded onto a Vydac C-4 reverse phase column (4.9 mm x 25 cm) equilibrated in 0.1% trifluoroacetate. The column was eluted with a linear gradient to 25% acetonitrile.

Mass spectral analysis of rat CTFP immunoreactivity. Mass spectrometry was performed with a JEOL HX100HF instrument operating at a 5kV accelerating potential, 3000 resolution, equipped with a 3 kV xenon atom source.

RESULTS AND DISCUSSION

A schematic diagram of rat preprogastrin is shown in Fig. 1. The signal peptide is cleaved away from progastrin as the peptide crosses the endoplasmic reticulum. The progastrin can be divided into four regions; the amino terminal flanking peptide, gastrin-34, the tripeptide Gly-Arg-Arg which presumably is the recognition region for the amidation of phenylalanine, and the carboxyl terminal flanking peptide (CTFP). The first three residues of rat, porcine and human CTFP are identical and the next four residues also are highly conserved. The human CTFP contains only six residues while porcine and rat contain nine residues. The last two residues of rat CTFP (Tyr-Asn) are different from porcine CTFP (Arg-Pro). It is not known if the tyrosine residue in rat is a potential sulfation site (11). Dockray has found that the amino terminal serine of CTFP is phosphorylated in pigs (7).

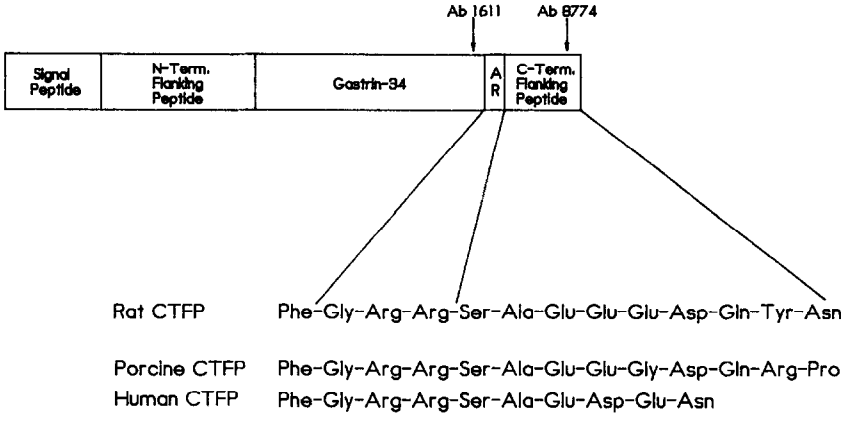

Figure 1. Schematic representation of rat preprogastrin based on cDNA sequence analysis (5). The signal peptide is 21 amino acids long. The amino terminal flanking peptide (N-Term. Flanking Peptide) is 37 amino acids long. Gastrin 34 can be cleaved to form smaller biologically active peptides (gastrin-17 or gastrin-14). The amidation region (AR) is -Gly-Arg-Arg- and signals for amidation of the phenylalanine of gastrin-34). The carboxyl terminal flanking peptide (C. Term. Flanking Peptide) begins with serine in rat (5), pig (2) and man (3,4) and ends with a different amino acid for the three species. The specificity of the antibodies used in this study are indicated by arrows. Antibody 1611 is specific for the carboxyl terminus of gastrin and antibody 8774 for rat CTFP.

A synthetic duplicate of rat CTFP (Fig. 1) was made so that a region specific radioimmunoassay could be developed. This nonapeptide has also been used as a standard for chromatographic comparison with natural peptides. Table 1 shows the amino acid analysis of the synthetic peptide and its mass

Table 1. Amino Acid Composition for Synthetic CTFP and Mass Spectral Analysis for Synthetic and Natural CTFP

	Gastrin-CTFP (residues / mole)	predicted value
Asp	2.0	2
Thr	0.0	
Ser	1.0	1
Glu	3.9	4
Pro	0.0	
Gly	0.0*	
Ala	1.0	1
Val	0.0	
Met	0.0	
Ile	0.0	
Leu	0.0	
Tyr	1.0	1
Phe	0.0	
His	0.0	
Lys	0.0	
Arg	0.0	
MH^+ calculated for CTFP		1084.41
MH^+ observed natural CTFP		1084.36
MH^+ observed synthetic CTFP		1084.11

* background levels (84 pmole) of glycine were observed.

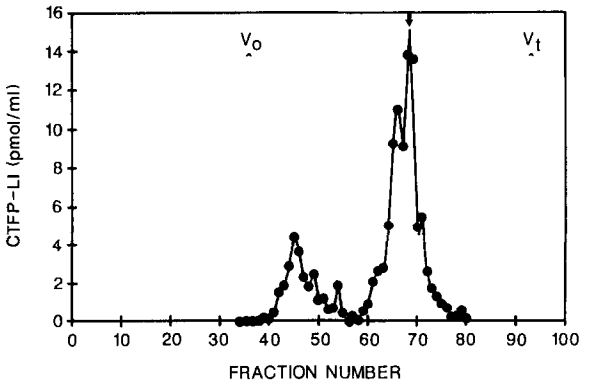

Figure 2. Elution profile of CTFP immunoreactivity (CTFP-LI) of an aqueous extract of rat antrum. The supernatant (2 ml) of the antral extract was loaded onto a Sephadex G-50 column (1 x 95 cm) equilibrated in sodium barbitol. Fractions (1 ml) were assayed for CTFP immunoreactivity. The void volume (V_0) and the total volume (V_t) were marked with radiolabeled albumin and $Na^{125}I$. The arrow indicates the region where synthetic CTFP elutes.

spectral analysis. The amino acid composition was consistent with the desired peptide. The molecular weight of the synthetic peptide the was the same as expected. The ID_{50} for antiserum 8771 was 20 fmol/ml for synthetic CTFP. This antiserum did not cross react with amidated gastrin-17 or glycine extended gastrin-6. This antiserum was used to detect CTFP immunoreactivity during its purification from rat antral extracts.

Figure 2 shows the analytical gel permeation profile of the CTFP immunoreactivity from rat antral extracts. Two major peaks of immunoreactivity were observed. The earlier eluting form of CTFP immunoreactivity may correspond to a larger form (i.e. gastrin-17 extended by the amidation region and CTFP). The later eluting form accounts for the majority of antral CTFP immunoreactivity. Synthetic CTFP eluted in the same region as the later eluting form. This co-elution was observed when the synthetic CTFP and antral extracts were eluted separately or together (data not shown).

The major peak of CTFP immunoreactivity also eluted last during preparative gel permeation chromatography (data not shown). This material was further purified by anion-exchange chromatography on a mono Q FPLC. Two peaks of CTFP immunoreactivity eluted from this anion-exchange column. The most prominent peak eluted in the same position as synthetic CTFP (Fig. 3A). This major peak of immunoreactivity was then purified on a reverse phase C-4 column. One peak of CTFP immunoreactivity eluted from this column (Fig. 3B) and it was associated with a single absorbance peak. This material eluted in the same region as synthetic CTFP which was run separately.

The molecular ion of the natural peptide was 1084, the same as the calculated value and the value obtained for synthetic CTFP. Thus, the major form of CTFP immunoreactivity was purified as the unmodified nonapeptide shown in figure 1. This peptide should be fully cross reactive in radioimmunoassays using antibody 8771.

The later elution position of the minor form of CTFP immunoreactivity during anion-exchange chromatography (Fig. 3A) suggests that it is a more negatively charged peptide than synthetic CTFP. Possible modifications that could cause this increased negative charge are sulfation of the penultimate tyrosine, phosphorylation of the amino terminal serine, or extension of the CTFP by gastrin-17. The

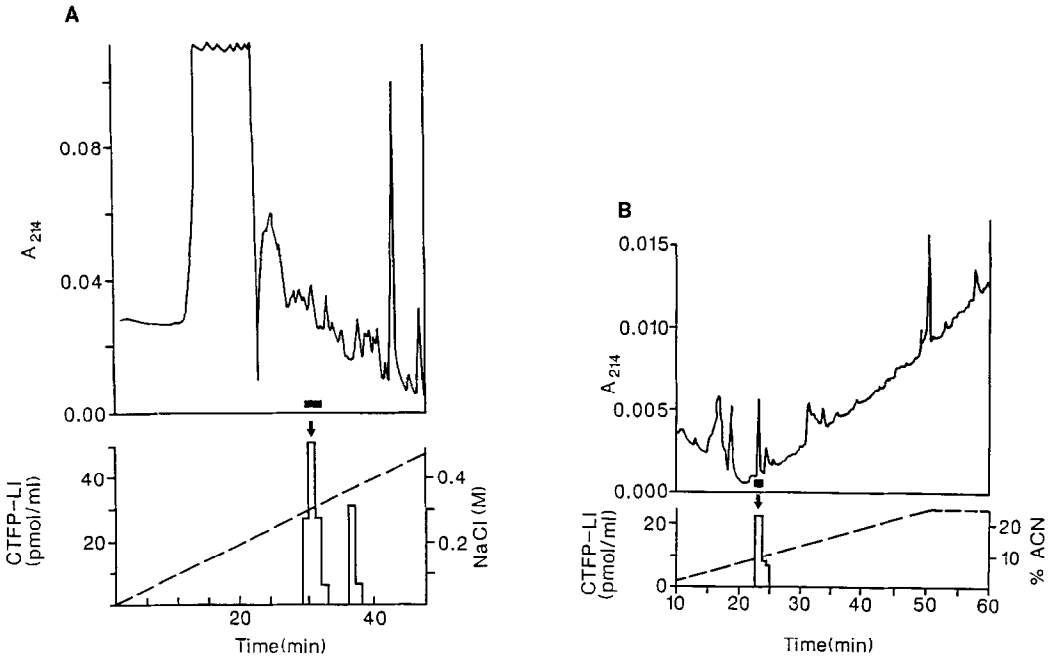

Figure 3. A. Elution profile of purified CTFP immunoreactivity (CTFP-LI) from Mono Q FPLC anion column. CTFP immunoreactivity eluting last from the preparative Sephadex G-50 column was pooled and loaded onto 3 separate Mono Q columns. Each column was eluted with increasing soncentrations of NaCl (- - -). This figure shows the elution profile from one of these columns. The absorbance at 214 nm (A_{214}) and CTFP-LI were monitored. The arrow indicates where synthetic CTFP elutes and the solid bar indicates the region pooled for reverse phase HPLC.

B. Elution profile of purified CTFP immunoreactivity from C-4 reverse phase HPLC. The major CTFP-LI from Mono Q was pooled (3A) and loaded onto an analytical C-4 column. The column was eluted with increasing concentrations of acetonitrile (- - -) and the absorbance at 214 nm (A_{214}) and CTFP-LI were monitored. The arrow indicates where the synthetic CTFP elutes and the solid bar indicates the region pooled for mass spectral analysis.

minor form of CTFP immunoreactivity was further purified on a reverse phase C-4 column. The height of the absorbance peak to immunoreactivity was similar to that observed for the peak of unmodified CTFP. However, the amounts of this more negatively charged CTFP immunoreactivity purified were too low to allow subsequent characterization. Its co-elution during gel permeation chromatography is most consistent with a sulfation or phosphorylation modification. The tyrosine in the proximity of several acidic groups has been suggested as potential site for sulfation (11). The serine has been shown to phosphorylated in porcine gastrin carboxyl terminal extension peptide (8). Whatever the modification, it apppears that it will not hinder immunological measurements of CTFP concentration since the absorbance and immunoreactivity were similar to the unmodified CTFP for antibody 8771.

Our data suggest that both of the immunoreactivities purified from the later eluting gel permeation peak are fully crossreactive. The earlier eluting immunoreactivity from gel permeation chromatography probably corresponds to CTFP extended with gastrin-17, gastrin-34 or even larger gastrin forms. This larger CTFP immunoreactivity has not yet been purified and chemically characterized. The structural characterization of the major forms of CTFP immunoreactivity will facilitate studies of processing of rat progastrin during various developmental or physiological states such as weaning and feeding.

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REFERENCES

1. Gregory,R.A., Tracy,H.J. (1964) The constitution and properties of two gastrins extracted from hog antral mucosa. Gut 5:103-117.
2. Yoo,O.J.,Powell,C.T.,and Agarwal,K.L. (1982) Molecular cloning and nucleotide sequence of full-length cDNA coding for porcine gastrin. Proc.Natl. Acad. Sci. USA 79:1049-1053.
3. Boel,E.,Vuust,J.,Norris,F.,Norris,K.,Wind,A.,Rehfeld,J.F. and Marker,K.A. (1983) Molecular cloning of human gastrin cDNA: evidence for evolution of gastrin by gene duplication. Proc.Natl.Acad.Sci. USA 80:2866-2869.
4. Kato,K.,Himeno,S.,Takahashi,Y.,Wakabayashi,T.,Tarui,S.,and Matsubara,K. (1983) Molecular cloning of human gastrin precursor DNA. Gene 26:53-57.
5. Fuller,P.J.,Stone,D.L.,and Brand,S.J. (1987) Molecular cloning and sequencing of a rat preprogastrin complementary deoxyribonucleic acid. Mol. Endocrinol. 1:306-311.
6. Reeve,J.R.,Dimaline,R. Shively,J.E.,Hawke,D. Chew,P. and Walsh,J.H. (1981) Unique amino terminal structure of rat little gastrin. Peptides 2:453-458.
7. Dockray,G.J.,Varro,A.,Desmond,H.,Young,J.,Gregory,H.,and Gregory,R.A. (1987) Post-translational processing of the porcine gastrin precursor by phosphorylation of the COOH-terminal fragment. J.Biol.Chem. 262:8643-8647.
8. Pauwels,S., Desmond,H.,Dimaline,R., and Dockray,G.J. (1986) Identification of progastrin in gastrinomas, antrum, and duodenum by a novel radioimmunoassay. J.Clin.Invest. 77:376-381.
9. Barany, G. and Merrifield, R.B. (1980) in **The Peptides, 2**, 1-284. Gross, E. and Meienhofer, J. Eds. Academic Press, New York
10. Hunter W.M. and Greenwood, F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature (London) 194: 495-496.
11. Huttner,W.B. (1987) Protein tyrosine sulfation. TIBS 12:361-363.