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Identification of a bioactive domain in the amino-terminus of glucose-dependent insulinotropic polypeptide (GIP)

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Abstract

The incretins are a class of hormones released from the small bowel that act on the endocrine pancreas to potentiate insulin secretion in a glucose-dependent manner. Due to the requirement for an elevated glucose concentration for activity, the incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1, have potential in the treatment of non-insulin-dependent diabetes mellitus. A series of synthetic peptide GIP fragments was generated for the purpose of elucidating the bioactive domain of the molecule. Peptides were screened for stimulation of cyclic AMP (cAMP) accumulation in Chinese hamster ovary cells transfected with the rat islet GIP receptor. Of the GIP fragments tested, GIP_{1–14} and GIP_{19–30} demonstrated the greatest cAMP-stimulating ability over the range of concentrations tested (up to 20 μM). In contrast, GIP fragments corresponding to amino acids 15–42, 15–30, 16–30 and 17–30 all demonstrated weak antagonism of GIP_{1–42} activity. Competitive-binding displacement studies indicated that these peptides were low-affinity ligands for the GIP receptor. To examine biological activity *in vivo*, a bioassay was developed in the anesthetized rat. Intravenous infusion of GIP_{1–42} (1 pmol/min/100 g) with a concurrent intraperitoneal glucose load (1 g/kg) significantly reduced circulating blood glucose excursions through stimulation of insulin release. Higher doses of GIP_{1–14} and GIP_{19–30} (100 pmol/min/100 g) also reduced blood glucose excursions. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Glucose-dependent insulinotropic polypeptide (GIP; gastric inhibitory polypeptide) and glucagon-

like peptide-1 (GLP-1) are considered to be the two most important incretins of the enteroinsular axis, where incretins are defined as gastrointestinal hormones which stimulate insulin release in a glucose-dependent manner [1,2]. Postprandial release of GIP and GLP-1 from the proximal and distal small intestine, respectively, accounts for the discrepancy between the oral and intravenous (i.v.) glucose-tolerance test insulin release profiles; the incretin effect

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accounting for approximately 50% of the postprandial insulin response [3,4].

The GIP receptor has been cloned, and found to belong to the class B family of heptahelical G protein-coupled receptors, that includes those for glucagon, secretin and vasoactive intestinal peptide (VIP) [5]. The GIP receptor is positively coupled to the adenylyl cyclase/cyclic adenosine monophosphate (cAMP) intracellular messenger cascade and is able to increase intracellular calcium accumulation by several mechanisms. It is thought that activation of the adenylyl cyclase pathway is the primary mode of action of GIP (reviewed in [6]).

Recently, GIP_{1–30} was shown to exhibit insulinotropic activity equivalent to that of GIP_{1–42} in the perfused rat pancreas, but to have significantly reduced somatostatinotropic activity in the isolated perfused rat stomach [7,8]. Moreover, a peptide fragment consisting of amino acids 19–30 of GIP's primary sequence was found to be sufficient to stimulate significant insulin release from the perfused pancreas [7]. Contrary to this finding, a body of evidence has accumulated signifying the importance of the amino-terminus for GIP action. Early studies identified GIP_{3–42} as a contaminant in natural porcine GIP preparations, which could be separated by high-performance liquid chromatography (HPLC), and did not possess biological activity [9]. This peptide was later established as the product of dipeptidyl peptidase IV (DPIV) cleavage of GIP [9,10]. Furthermore, GIP_{4–42} retained high-affinity receptor binding, but lacked biological activity and was proposed to behave as a receptor antagonist [11]. These studies were extended by Gelling et al. [12], showing that further truncation of the amino-terminus resulted in peptides with antagonist activity.

Hence, it was hypothesized that the amino-terminus was a greater determinant of biological activity, and perhaps study of N-terminal peptide analogs would yield shorter length bioactive peptides. A series of amino- and carboxyl-terminal synthetic GIP fragments was generated and tested on Chinese hamster ovary (CHO-K1) cells transfected with the wild-type rat pancreatic GIP receptor (wtGIPR cells). Biological activity of the fragments was assessed through measurement of intracellular cAMP in transfected cells, insulin release from the perfused rat pancreas, and with an *in vivo* bioassay, while

binding affinity was assessed through competitive-radioligand binding assays.

2. Materials and methods

2.1. Cell transfection and tissue culture

CHO-K1 cells were transfected with 10 µg of GIP receptor cDNA in pCDNA3 (Invitrogen, Carlsbad, CA, USA) by the calcium phosphate method; the stable cell line wtGIPR (formerly named rGIP-15) was established by clonal isolation, and characterized to express 12 000–15 000 receptors per cell [13]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco BRL, Burlington, ON, Canada), supplemented with 10% newborn bovine serum (Cansera, Rexdale, ON, Canada), and antibiotics (Sigma-Aldrich, Oakville, ON, Canada): penicillin G (50 U/ml) and streptomycin (50 µg/ml). Cells were maintained under high selection with 800 µg/ml G418 (Gibco). Cells were cultured in 75-cm² T-flasks (Falcon, Becton Dickinson, Mississauga, ON, Canada) until 80–90% confluent, prior to harvesting for use in binding or cAMP studies. Cells were removed from the culture flask with trypsin/EDTA, seeded into 24-well plates (Falcon) at 50 000 cells per well, and allowed to grow for 48 h (until approximately 90% confluent) before use in experiments.

2.2. Peptides

Synthetic human and porcine GIP_{1–42} were purchased from Bachem (Torrance, CA, USA). Peptide fragments were synthesized with an automated synthesizer, Symphony (Rainin Instrument Co., Woburn, MA, USA), using a modified Fmoc protocol. All reagents for peptide synthesis were analytical grade, and obtained from Novabiochem (Schwalbach, Germany), Roth (Karlsruhe, Germany) or J.T. Baker (Griesheim, Germany). Peptides were purified to > 95% purity by reverse-phase (RP)-HPLC; identity and purity of peptides was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Peptide synthesis methods, as applied to synthesis of glucagon fragments and analogs, are described in detail elsewhere [14].

2.3. Receptor-binding studies

The binding study protocol used in this report has been previously described [15]. Synthetic porcine GIP (Bachem, Torrance, CA, USA) was iodinated by the chloramine T method, and purified by RP-HPLC such that the resulting labelled peptide was iodinated only on Tyr¹⁰; the specific radioactivity of the ¹²⁵I-GIP was approximately 250–350 μ Ci/mg as described in detail previously [10]. Cells prepared 48 h previously, having grown to a density of approximately $3\text{--}5 \times 10^5$ cells/well in 24-well plates, were first washed twice with ice-cold assay buffer (DMEM/F12, 15 mM HEPES (Sigma-Aldrich), 0.1% bovine serum albumin (BSA; Sigma-Aldrich), pH 7.4). Binding competition assays were then performed in the same buffer supplemented with 1% Trasylol (aprotinin; Bayer, Etobicoke, ON, Canada). ¹²⁵I-GIP (50 000 cpm) was allowed to compete with unlabeled synthetic human GIP or GIP fragments for 12–16 h at 4°C. After incubation, cells were again washed twice with ice-cold assay buffer, solubilized with 0.1 M NaOH and counted in a γ counter. Non-specific binding was defined as the amount of cell-associated radioactivity measured from the assay wells containing excess GIP_{1–42} (1 μ M).

2.4. cAMP studies

Cells were washed twice with 37°C HEPES-buffered DMEM/F12 supplemented with 0.1% BSA (assay buffer; defined above) and allowed to pre-incubate in assay buffer at 37°C for 1 h prior to the stimulation period. Cells were stimulated for 30 min in assay buffer supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (Research Biochemicals Int., Natick, MA, USA) and peptide concentrations indicated in figures. For antagonism experiments, cells were first incubated with peptides for 15 min prior to a 30-min stimulation with 1 nM GIP_{1–42}, or 20 μ M GIP_{1–14} or GIP_{19–30} as indicated. Intracellular contents were extracted with ice-cold 70% ethanol (1 ml/well), cellular debris was separated by centrifugation (5 min, 4°C, 10 000 rpm) and samples were dried (Speed Vac, Savant, Farmingdale, NY, USA). Samples were reconstituted in sodium acetate buffer (0.05 M, pH 6.2) and cAMP was measured by radioimmunoassay as per the manufacturer's instruc-

tions (Biomedical Technologies, Stoughton, MA, USA). cAMP data are expressed in fmol/1000 cells or as fold-basal. The cAMP stimulation and antagonism protocols are based upon previous research techniques [12].

2.5. Animals

Male Wistar rats were obtained from the University of British Columbia Animal Care Facility (Vancouver, BC, Canada). Animals were maintained in group housing conditions with free access to rat food and water, and under a 12-h light/dark cycle. Rats were fasted for 15–18 h prior to experimentation. Anesthesia was induced by intraperitoneal (i.p.) injection of 65 mg/kg sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada). All animal experiments are in accordance with the guidelines put forth by the University of British Columbia Committee on Animal Care and the Canadian Council on Animal Care.

2.6. Pancreas perfusions

Isolation and perfusion of pancreases from anesthetized fasted male Wistar rats (250–300 g) were performed as previously described [7,16,17]. The perfusate was Krebs–Ringer bicarbonate buffer (pH 7.4) with 3% dextran, 0.2% BSA and 16.7 mM glucose, with or without synthetic GIP or fragments. Peptides were perfused over a linear gradient of 0–5 nM according to published methods [7,16]. Effluent samples were collected from the portal vein and assayed for insulin content by radioimmunoassay. Briefly, appropriately diluted samples and rat insulin standards (Novo Nordisk, Denmark) were incubated with antibody GP01 (1:1 000 000 dilution) for 24 h, prior to addition of 2000 cpm ¹²⁵I-insulin prepared by chloramine-T iodination. Twenty-four hours after addition of label, bound and free antigen were separated using dextran-coated charcoal and centrifugation. This radioimmunoassay has been described in more detail elsewhere [7,16].

2.7. Bioassay

Cannulae were inserted into the jugular vein and the carotid artery of anesthetized fasted male Wistar

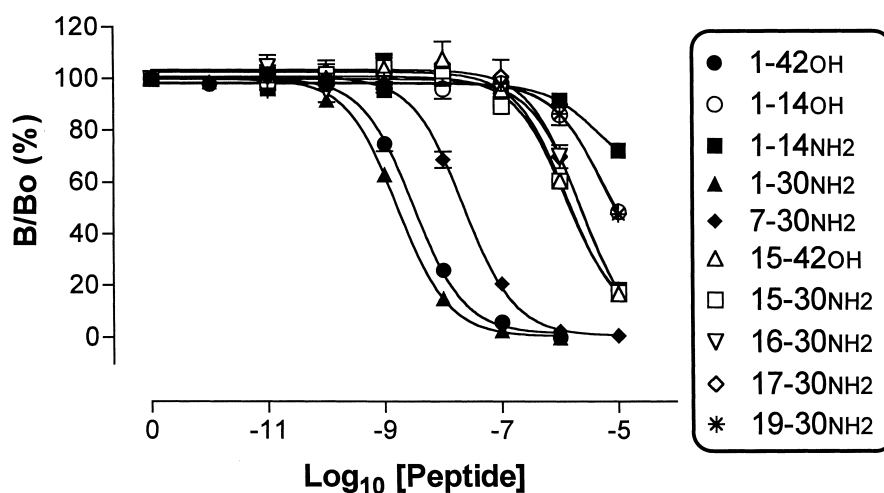


Fig. 1. Competition-binding displacement curves of synthetic GIP fragments and ^{125}I -GIP $_{1-42}\text{OH}$ on CHO-K1 cells transfected with the rat GIP receptor (wtGIPR cells). Data represent the mean \pm S.E.M. of 3–8 independent experiments. Refer to Table 1 for binding statistics.

rats (150–250 g). Basal blood samples were withdrawn from the carotid artery (500 μl), and fasted blood glucose was measured via the tail vein using a SureStep glucose analyzer (LifeScan Canada, Burnaby, BC, Canada). I.v. saline or peptide infusion (2.3 ml/h) was then started ($t = -5$ min). At $t = 0$ min, an i.p. glucose injection was given (1 g/kg b.wt.). Blood samples were taken at 10, 20, 30 and 60 min, and blood glucose was monitored at 10-min intervals throughout the experiment. Plasma was separated by centrifugation (10 000 rpm, 10 min, 4°C) and immunoreactive insulin was measured by radioimmunoassay, as described above.

2.8. Data analysis

Data are expressed in terms of mean \pm S.E.M. with the number of independent experiments in brackets. Each independent experiment for binding and cAMP studies consisted of triplicate determinations; insulin radioimmunoassay samples were measured in duplicate. Binding and cAMP data were analyzed with the non-linear regression analysis program PRISM (Graphpad, San Diego, CA, USA). Integrated insulin responses were calculated using the trapezoidal method, with basal insulin release as the baseline, using the algorithm included in the PRISM software package. In order to determine significance of difference in comparisons, where appropriate, the data was first subjected to analysis of variance and then

a post hoc test, Dunnett's Multiple Comparison Test, was used to compare the stimulated responses to the control response.

3. Results

3.1. Competitive-binding studies

Binding affinities of synthetic peptides were determined by binding-displacement assays on CHO-K1 cells transfected with wtGIPR cells (Fig. 1). A summary of statistics is shown in Table 1. Of the peptides tested, only GIP $_{1-42}$, GIP $_{1-30}\text{NH}_2$ and GIP $_{7-30}\text{NH}_2$ were able to fully displace ^{125}I -GIP binding (IC_{50}s : 3.17 ± 0.3 , 2.04 ± 0.73 and 23.7 ± 3.7 nM, respectively; $n = 4$). Truncation of the amino-terminus by 14, 15 and 16 amino acids resulted in C-terminal fragments (GIP $_{15-30}\text{NH}_2$, GIP $_{16-30}\text{NH}_2$ and GIP $_{17-30}\text{NH}_2$) that produced half-maximal displacement values in the low micromolar range (Table 1); further truncation at the amino-terminus resulted in IC_{50}s greater than 10 μM , and thus cannot be determined from the range of peptides tested. Short amino-terminal peptides were less potent at displacing specific ^{125}I -GIP binding: GIP $_{1-14}\text{OH} >$ GIP $_{1-14}\text{NH}_2 >$ GIP $_{1-13}\text{NH}_2 =$ GIP $_{1-15}\text{NH}_2$ (Fig. 1, Table 1). Peptides GIP $_{1-6}\text{NH}_2$, GIP $_{1-7}\text{NH}_2$, GIP $_{1-13}\text{OH}$ and GIP $_{1-15}\text{OH}$ failed to displace significant ^{125}I -GIP under the given assay conditions (Table 1).

Table 1

Summary statistics for cAMP production and competitive-binding displacement studies on synthetic GIP fragments using CHO-K1 cells transfected with the rat GIP receptor

Synthetic peptide	Molecular weight (Da)		cAMP production (fold-basal ^a)		Receptor binding	
	expected	measured	10 μ M	20 μ M	% displacement at 10 μ M	IC ₅₀ (nM)
GIP(1–42OH)	4984.3	NA ^b	119 \pm 11 ^{c,d}	– ^c	100 ^c	3.2 \pm 0.3
1–6NH ₂	685.7	686.9	1.27 \pm 0.18	1.08 \pm 0.03	–3.6 \pm 7.8	–
1–7NH ₂	798.9	800.2	0.92 \pm 0.05	1.06 \pm 0.06	–6.1 \pm 3.4	–
1–13OH	1436.5	1438.2	1.03 \pm 0.06	1.15 \pm 0.07	–0.2 \pm 3.4	–
1–13NH ₂	1435.6	1435.6	6.51 \pm 1.33 ^d	15.7 \pm 3.0 ^d	5.0 \pm 1.1 ^d	–
1–14OH	1567.8	1569.3	88.9 \pm 9.5 ^d	85.2 \pm 7.6 ^d	51.3 \pm 1.2 ^d	–
1–14NH ₂	1566.8	1569.7	75.4 \pm 10.7 ^d	88.3 \pm 5.9 ^d	27.9 \pm 2.8 ^d	–
1–15OH	1682.8	1680.3	0.97 \pm 0.06	0.91 \pm 0.05	–3.1 \pm 4.3	–
1–15NH ₂	1681.9	1682.6	2.26 \pm 0.32 ^d	4.37 \pm 0.51 ^d	4.2 \pm 1.7 ^d	–
1–30NH ₂	3552.0	3553.3	108 \pm 12 ^{c,d}	– ^c	99.8 \pm 1.2 ^d	2.0 \pm 0.7
7–30NH ₂	2882.3	2886.9	0.89 \pm 0.06	0.85 \pm 0.03	99.3 \pm 1.0 ^d	23.7 \pm 3.7
15–42OH	3433.8	3434.4	1.02 \pm 0.10	1.01 \pm 0.03	83.3 \pm 0.7 ^d	1270 \pm 150
15–30NH ₂	2001.3	2003.3	1.24 \pm 0.28	1.01 \pm 0.11	82.7 \pm 1.0 ^d	1400 \pm 310
16–30NH ₂	1886.3	1887.6	1.04 \pm 0.06	0.80 \pm 0.02	82.1 \pm 1.9 ^d	2530 \pm 450
17–30NH ₂	1758.1	1761.1	1.13 \pm 0.09	1.12 \pm 0.05	81.9 \pm 2.1 ^d	1540 \pm 550
19–30NH ₂	1488.7	1489.8	20.1 \pm 1.3 ^d	45.0 \pm 1.6 ^d	52.3 \pm 0.6 ^d	–

Data represent mean \pm S.E.M. of at least three independent experiments. Refer to text for specific methods.

^a Basal cAMP = 2.96 \pm 0.03 fmol/1000 cells.

^b GIP_{1–42} was purchased from Bachem (Torrance, CA, USA).

^c cAMP and binding experiments with GIP_{1–42} and GIP_{1–30NH₂} were only tested at concentrations as high as 1 μ M.

^d $P < 0.05$ versus control conditions, denoting significant increase in cAMP over basal levels or significant displacement of ¹²⁵I-GIP.

3.2. Cyclic AMP studies

All peptides were screened on wtGIPR cells initially with a concentration–response curve over the

range of 1 pM to 20 μ M. Table 1 shows the summary of peptides tested for the 10 and 20 μ M responses; peptides showing no cAMP stimulation at high concentration did not demonstrate any activity

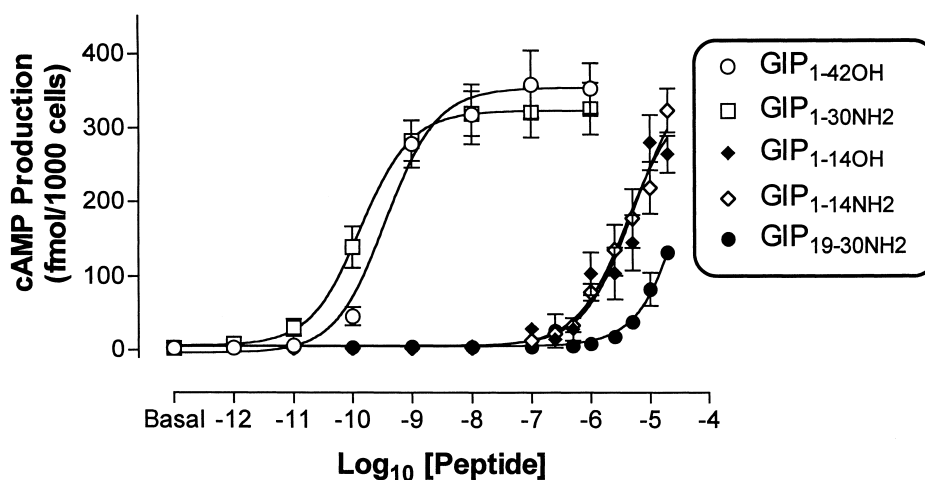


Fig. 2. Concentration–response curves of intracellular cAMP production in wtGIPR cells by synthetic GIP and selected bioactive truncated peptides. Data represent mean \pm S.E.M. of at least three independent experiments. Refer to Table 1 for cAMP data for all GIP peptides tested.

at lower concentrations (data not shown). The basal cAMP level in wtGIPR cells was 2.96 ± 0.03 fmol/1000 cells ($n > 30$). Of peptides tested, only GIP_{1–14} (amidated or free acid) and GIP_{19–30} produced large cAMP responses. GIP_{1–13NH₂} and GIP_{1–15NH₂} also showed significant increases in cAMP from basal levels, however, responses were weak compared to GIP_{1–14}. Full concentration–response curves for bioactive GIP fragments are shown in Fig. 2. GIP_{1–42} had an EC₅₀ of 377 ± 25 pM for cAMP production, and maximal cAMP generation was 358 ± 46 fmol/1000 cells ($n = 6$); similarly, GIP_{1–30NH₂} had an EC₅₀ of 139 ± 77 pM for cAMP production, and maximal cAMP generation was 326 ± 35 fmol/1000 cells ($n = 3$). GIP_{1–14} and GIP_{19–30} did not stimulate maximal cAMP levels over the concentration range tested, and thus it is difficult to estimate EC₅₀ values. However, given the maximal cAMP production by GIP_{1–42}, the EC₅₀ of GIP_{1–14} (C-terminal amide or free acid) was in the micromolar range, and both amidated and hydroxylated forms of GIP_{1–14} appear to be full agonists of the receptor (Fig. 2). Bioactive GIP fragments had no effect on non-transfected CHO-K1 cells (data not shown).

GIP fragments truncated at the amino-terminus have previously been shown to act as potent receptor antagonists [12], and thus the shorter C-terminal fragments were similarly tested for inhibition of GIP-stimulated cAMP production (Fig. 3A). The potency of GIP fragments as antagonists paralleled their binding affinities (Table 1). Peptides GIP_{15–42OH}, GIP_{15–30NH₂}, GIP_{16–30NH₂} and GIP_{17–30NH₂} acted as weak antagonists of the GIP receptor; GIP_{7–30NH₂} was included as a positive control [12].

To further delineate structure–activity relationships of GIP, and as an important control, the ability of GIP_{17–30NH₂} to antagonize GIP_{1–14OH} and GIP_{19–30NH₂} was examined. It was hypothesized that the non-overlapping C-terminal GIP_{17–30NH₂} fragment would not antagonize GIP_{1–14OH}, whereas it should antagonize GIP_{19–30NH₂}. The results for these experiments are found in Fig. 3B,C (note: data were normalized to 20 μM concentrations of agonist for better comparison, as GIP_{1–14OH} is more potent than GIP_{19–30NH₂}; see Fig. 2). In confirmation of the proposed hypothesis, 20 μM GIP_{17–30NH₂} was not able to significantly reduce cAMP stimulated by 20 μM GIP_{1–14OH}, whereas

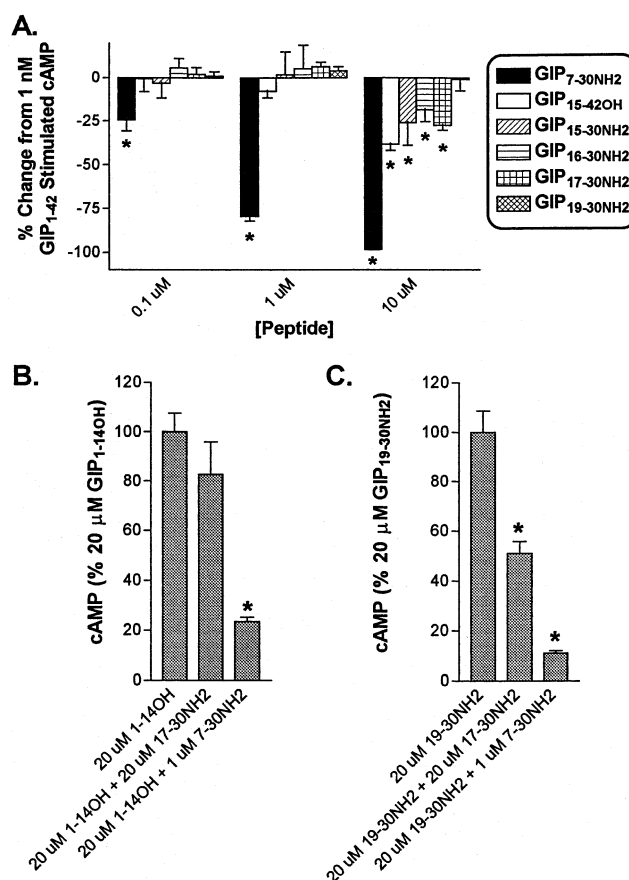


Fig. 3. (A) Antagonism of 1 nM GIP_{1–42} stimulated cAMP production in wtGIPR cells by N- and C-terminally truncated GIP fragments. On average, 1 nM GIP_{1–42} produced 278 ± 32 fmol of cAMP/1000 cells (cf. Fig. 2). Antagonism of (B) 20 μM GIP_{1–14OH} or (C) 20 μM GIP_{19–30NH₂} by 20 μM GIP_{17–30NH₂} or 1 μM GIP_{7–30NH₂}. Data represent mean \pm S.E.M. of > 3 independent experiments; * $P < 0.05$ versus control (1 nM GIP_{1–42} or 20 μM GIP_{1–14OH} or GIP_{19–30NH₂}).

1 μM GIP_{7–30NH₂} reduced this amount by $76.6 \pm 1.9\%$ ($P < 0.05$). Furthermore, 20 μM GIP_{17–30NH₂} reduced 20 μM GIP_{19–30NH₂}-stimulated cAMP by $48.9 \pm 4.7\%$ and 1 μM GIP_{7–30NH₂} inhibited by $89.0 \pm 1.1\%$ ($P < 0.05$).

3.3. Perfused rat pancreas and in vivo bioassay

Given the ability to stimulate cAMP, GIP_{1–14} was selected for testing in the perfused rat pancreas. The effect of GIP_{19–30} on insulin secretion in the perfused pancreas has already been tested [7]. A peptide gradient from 0–5 nM under high glucose conditions (16.7 mM) was perfused over 40 min. Immunoreac-

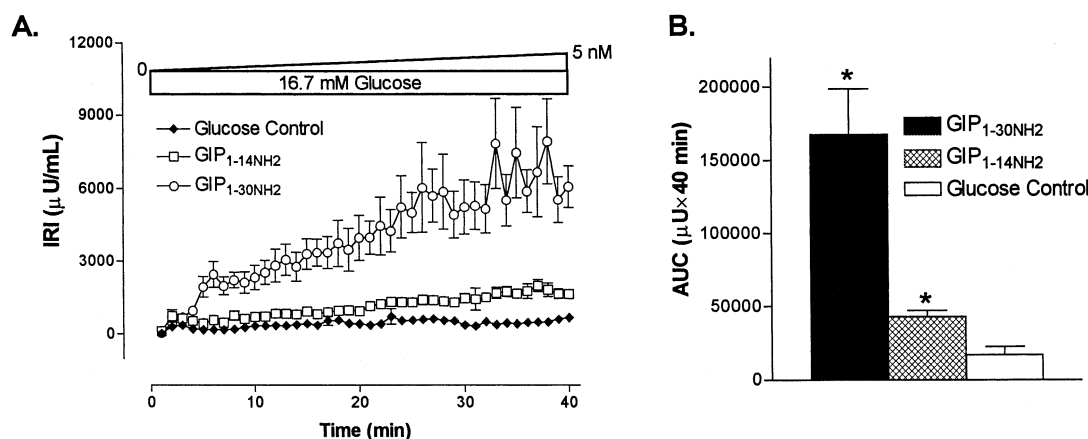


Fig. 4. (A) Immunoreactive insulin release from the perfused rat pancreas in response to 16.7 mM glucose, with or without linear peptide gradients of $\text{GIP}_{1-30\text{NH}_2}$ or $\text{GIP}_{1-14\text{NH}_2}$ (0–5 nM). (B) Integrated insulin responses for the data shown in (A). Data represent mean \pm S.E.M. of four experiments; * $P < 0.05$ versus glucose control.

tive insulin release profiles are shown for $\text{GIP}_{1-30\text{NH}_2}$ and $\text{GIP}_{1-14\text{NH}_2}$ as well as the glucose control (Fig. 4). GIP_{1-14} was found to induce a small, but significant, increase in insulin release ($P < 0.05$). Integrated responses for insulin release from the perfused pancreas were: $\text{GIP}_{1-30\text{NH}_2}$, 167.5 ± 62.7 mU; $\text{GIP}_{1-14\text{NH}_2}$, 43.0 ± 8.5 mU; glucose control, 17.1 ± 9.3 mU, over the 40 min perfusion period ($n = 3-4$).

A bioassay was developed based on the principal that GIP acts to lower blood glucose mainly through an insulin-dependent mechanism. Hence, blood glucose was monitored during peptide infusion following an i.p. glucose-tolerance test. Infusion of 1 pmol/min/100 g b.wt. of $\text{GIP}_{1-42\text{OH}}$ significantly reduced circulating glucose levels relative to control animals (Fig. 5A). Concurrent insulin measurements revealed that $\text{GIP}_{1-42\text{OH}}$ infusion induced a rapid peak in circulating insulin within 10 min of glucose injection, followed by a return to baseline at 60 min. In contrast, saline control animals demonstrated a slow rise in insulin, peaking at 30 min, and similarly return to baseline at 60 min (Fig. 5B). Because of the sampling times used, it was not possible to determine if GIP treatment enhanced phase I versus phase II insulin release, or both; however, recently it was demonstrated that GIP primarily reduces postprandial glucose excursions via augmenting the early phase of insulin release [18]. Synthetic GIP peptides shown to have biological activity on transfected cells and in the perfused pancreas were tested, monitoring

only blood glucose. $\text{GIP}_{1-42\text{OH}}$ and $\text{GIP}_{1-30\text{NH}_2}$ were equally effective in reducing excursions in glycemia, relative to saline controls (Fig. 6; $P < 0.05$ at all time points). A much greater dose of $\text{GIP}_{1-14\text{NH}_2}$ (100 pmol/min/100 g b.wt.) was required to achieve the same effect ($P < 0.05$ relative to controls at all time points), whereas the same dose of $\text{GIP}_{19-30\text{NH}_2}$ only slightly reduced the glucose response after i.p. glucose (Fig. 6; $P < 0.05$ at 20- and 30-min time points).

4. Discussion

The explicit prerequisite for incretin-induced insulin release is the need for hyperglycemic conditions. Thus, unlike other non-endogenous insulinotropic agents used in the treatment of type II diabetes, the incretins are unable to act inappropriately to stimulate insulin release during euglycemia. It is this unique feature which has led to recent interest in the incretins as a novel therapy for diabetes. Clinical trials have been restricted to GLP-1 [19], but administration of peptide analogs of both GLP-1 [19] and GIP [20,21] with prolonged circulating half-lives, and inhibition of DPIV [22–24], a physiological regulator of incretin activity, have both been shown to produce improved glucose tolerance in experimental models. Although some populations of type II diabetic patients have been reported to show decreased responsiveness to GIP, while responses to GLP-1 were un-

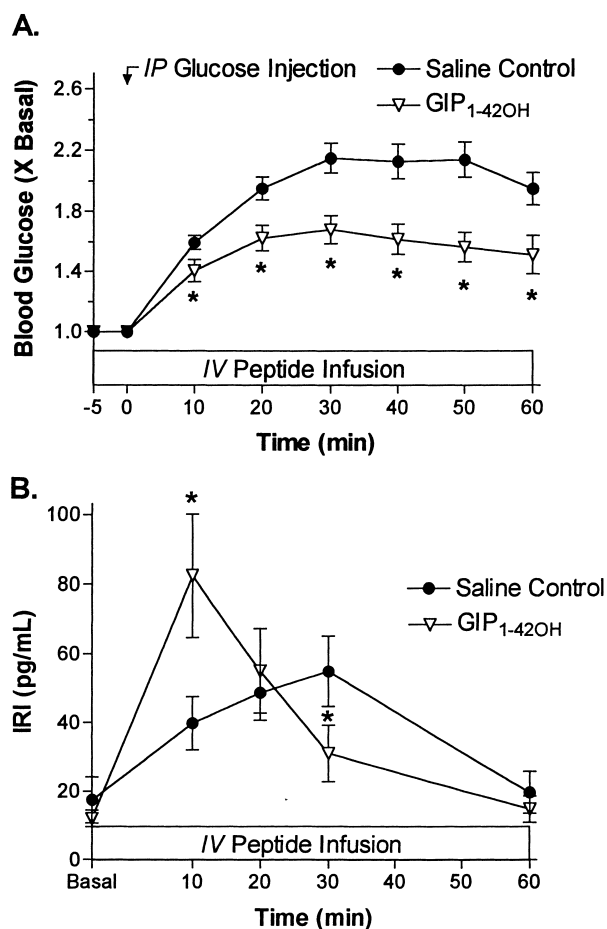


Fig. 5. Glucose (A) and immunoreactive plasma insulin (B) in anesthetized male Wistar rats on i.v. infusion of saline or GIP₁₋₄₂, with concurrent i.p. glucose challenge (1 g/kg). Data represent mean \pm S.E.M. of four animals; * $P < 0.05$ versus saline control.

affected [25–27], β -cell sensitivity to GIP improves with glyburide treatment [28]. Therefore, GIP analogs may be useful in the treatment of diabetes, and it is important to develop a full understanding of the mode of action of GIP.

In the last three decades, considerable effort has been targeted at the structural determination of small peptide hormones. Because short peptides are in general flexible, conformation depends on peptide concentration, solvent composition and other molecules present in solution [29]. X-ray diffraction studies have been limited to glucagon, although these studies compare well with solution structure determinations [29]. Solution structure determination techniques, circular dichroism and nuclear magnetic resonance

(NMR), have been performed on most members of the glucagon/secretin/VIP family [29–34], with the exception of GIP, peptide histidine isoleucine and glucagon-like peptide-2. In order to stabilize secondary structure, all studies have been performed in the presence of organic solvents or micelles, as there was little evidence of a stable structure in water alone [29,30]. The general structural features of the glucagon superfamily appear to be a disordered N-terminal region of 6–8 amino acids, followed by a helical region of 18 ± 2 amino acids, that can be a continuous helix, or broken into two segments by a hinge region of two amino acids [29–34]. The exendin peptides from *Helodermatidae* venom represent a family of peptides structurally related to the glucagon superfamily [35]. Structural determination has been completed for helodermin (exendin-2), which acts as an agonist at VIP and secretin receptors, indicating that it retains significant secondary structure in water, and this is enhanced by organic solvents [36]; helodermin has a core α -helix of 15 amino acids in water, and this extends to 21 amino acids with the addition of trifluoroethanol. It is thought that a helical structure is the preferred conformation for receptor binding, since changing the experimental conditions from aqueous to organic approximately mimics the situation in vivo for a blood-borne hormone going from solution to a membrane-bound receptor–hormone complex [36]. Notably, the related *Helodermatidae* venom peptide exendin-4 (a GLP-1 receptor agonist) and exendin-4_(9–39)NH₂ act as weak GIP receptor antagonists [13,37,38]. A long-term goal of structural studies is to compare NMR solution structures and effects of peptide deletions and substitutions on hormone bioactivity to determine whether there are common or specific structural features relevant to the modes of peptide action [36]. To date, GIP structural analysis has been limited to structure–function relationships using enzyme or chemically cleaved peptides or synthetic peptides and rudimentary computational methods (Fig. 7 and described below).

The current body of experimental evidence indicates that there are four dissociable domains in GIP₁₋₄₂. The high-affinity binding domain, GIP_{6–30}NH₂, is a potent GIP receptor antagonist [12]. Initially, two bioactive domains in GIP were indicated. Truncation of 12 amino acids from the

carboxyl-terminus of GIP_{1-42} resulted in a peptide with equivalent insulinotropic activity, but lacking somatostatinotropic activity [7,39]. Part of the GIP molecule in the carboxyl-terminus is therefore critical for its acid inhibitory (enterogastrone) activity [7,39,40]. However, it is unclear whether this is due to the existence of a second GIP receptor, an alternatively spliced receptor, or differential ligand recognition or coupling of the existing receptor in gastric cells. The insulinotropic domain of GIP was localized to residues 19–30, consistent with partial retention of insulinotropic activity of GIP_{19-30} , GIP_{15-42}

and GIP_{17-42} [7,11,17]. However, this was inconsistent with the importance of the amino-terminus in GIP signal transduction and regulation of GIP activity by DPIV [41]. In following this hypothesis, evidence presented here suggests a third bioactive domain of GIP, residing in residues 1–14 (Figs. 2, 4 and 6, and Table 1).

Computer-assisted secondary structure analysis of GIP predicts an α -helical region between residues 10 and 29 (Fig. 7; PCGENE, IntelliGenetics; GGBS Method [42]). Bioactivity of N-terminal GIP fragments was limited to GIP_{1-14} (amide or free acid)

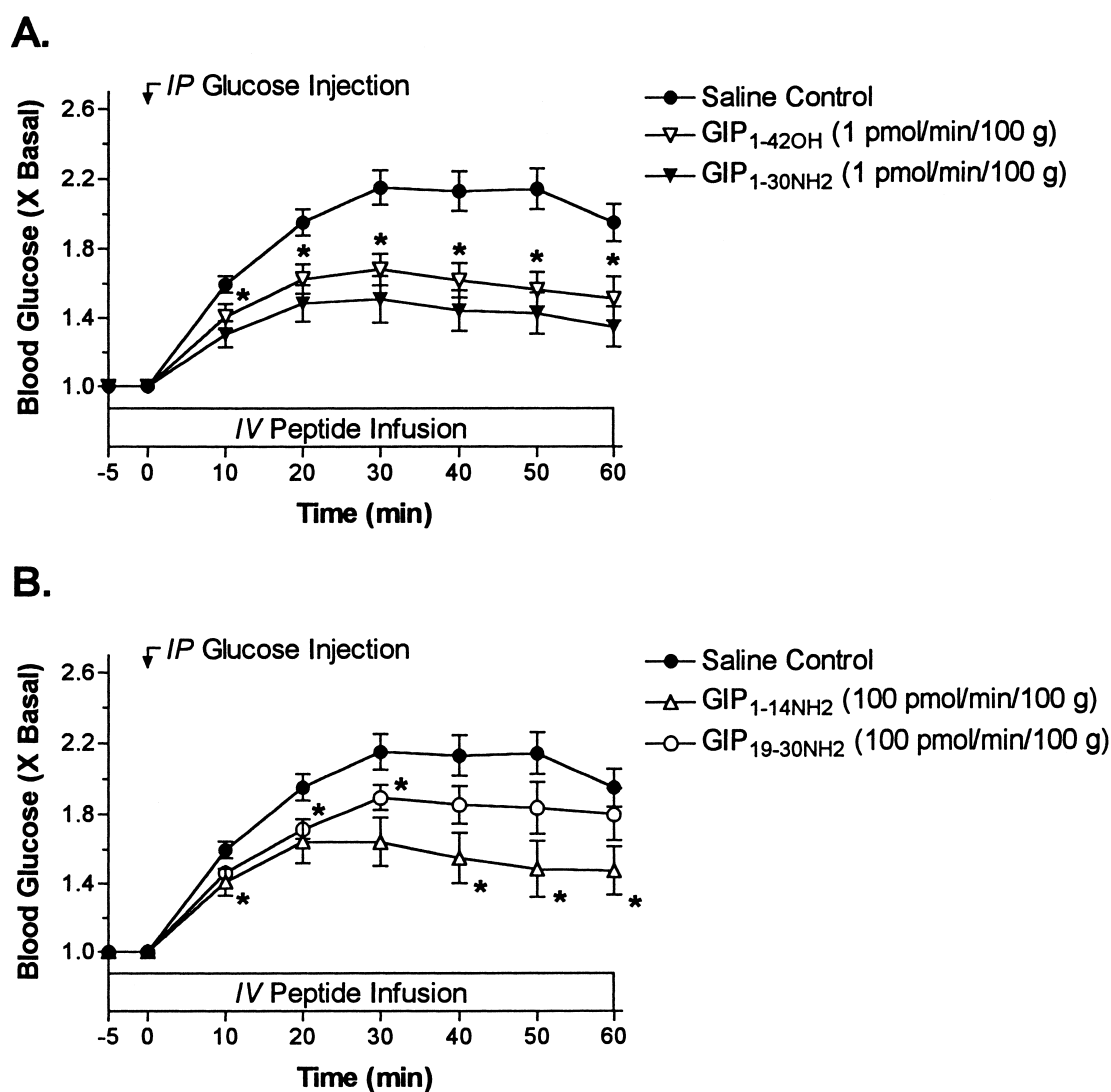


Fig. 6. Glucose-lowering effects of GIP analogs in anesthetized rats. (A) Peptides infused at a dosage of 1 pmol/min/100 g b.wt. versus saline control. (B) Peptides infused at a dosage of 100 pmol/min/100 g b.wt. versus saline control. Peptides in phosphate-buffered saline were infused intravenously, while glucose was administered by i.p. injection. Glucose was monitored by tail vein measurements using a SureStep blood glucose analyzer. Data represent mean \pm S.E.M. of four animals; * $P < 0.05$ versus saline control.

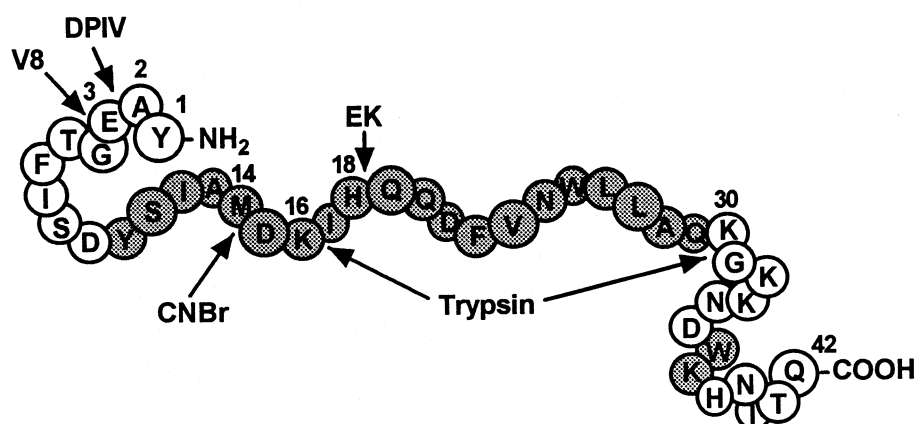


Fig. 7. Primary structure of GIP_{1-42} with predicted α -helical regions (gray; Gascuel and Golmard Basic Statistical Method [42]) and enzymatic cleavage sites used for structure–function relationships. Amino acid positions are indicated above the residues prior to cleavage sites. Abbreviations and references: DPIP, dipeptidyl peptidase IV [9,55]; V8, *Staphylococcus* V8 protease [11]; CNBr, cyanogen bromide [17,40]; trypsin [7,11]; EK, enterokinase [7].

and amidated forms of GIP_{1-13} and GIP_{1-15} . Hence, it is possible that, along with an intact amino-terminus ($\text{Tyr}^1\text{-Ala}^2$), preservation of this helical structure is also important for biological activity. In the absence of Met^{14} the helix may be unable to form, but the charged residue Asp^{15} destabilizes it. Amidation of the carboxyl-terminus appears to have minor effects on bioactivity, only noticeable for $\text{GIP}_{1-13}\text{NH}_2$ and $\text{GIP}_{1-15}\text{NH}_2$, where it may partially stabilize the secondary structure (Table 1). Contrary to data in the current report, GIP_{1-14} created by cyanogen bromide cleavage was not insulinotropic in the perfused rat pancreas [17]. However, conversion of Met^{14} to homoserine lactone by this cleavage method, when this region appears particularly sensitive to structural perturbations, likely generates a biologically inactive N-terminal peptide.

Study of the bioactivity of amino-terminal peptide fragments of the secretin–glucagon family is not limited to GIP. Data now exists for parathyroid hormone (PTH), secretin, glucagon and GLP-1 and VIP; however, bioactivity of these fragments appears to be largely dependent on the peptides examined. In a recently published report, micromolar concentrations of PTH_{1-14} were able to stimulate cAMP production in cells transfected with the human or rat PTH-1 receptors [43]. While it was not possible to demonstrate ^{125}I - PTH_{1-34} displacement by PTH_{1-14} using radioligand binding assays [43], photoaffinity cross-linking experiments have indicated that the

amino-terminal region of PTH interacts with the PTH receptor in the area of the junction of the extracellular tail and the first transmembrane domain [44–46]. Further work of Gardella et al. found that secretin_{1-13} activated the transfected secretin receptor [43]. However, in the course of our experiments, we have also tested $\text{glucagon}_{1-14}\text{OH}$ and $\text{GLP-1}_{[7-20]\text{OH}}$ on CHO-K1 cells transfected with human isoforms of their respective receptors, but neither peptide showed any stimulation of cAMP production at concentrations as high as $20\ \mu\text{M}$ (data not shown). $\text{GLP-1}_{[7-20]}$ was previously tested in the perfused rat and canine pancreas, and had little or no effect [47,48]. Carboxyl-terminal truncation of glucagon dramatically reduced bioactivity [49], and the shortest glucagon fragment reported to retain receptor binding and activation has been glucagon_{1-17} [50]. Synthetic VIP_{1-14} has also been reported to be inactive [51]. Thus, it appears that structural similarities of amino-termini across all members of the secretin/VIP/glucagon superfamily of hormones do not necessarily confer biological activity.

While structural data for GIP are lacking, it is possible to propose a mechanistic model for ligand binding and receptor activation. A large body of evidence has accumulated regarding receptor–ligand interactions for small ligands, including small peptides [52], however, the types of analyses performed on small ligands, such as thyrotropin (pyroGlu-His-ProNH₂) are not possible for polypeptide ligands

due to their complexity. Use of chimeric ligands and receptors has made it somewhat possible to dissect the molecular domains of peptide hormones necessary for binding and activation. Based on some of this work, Hjorth and Schwartz proposed production of a pseudo-tethered intermediate involving the large extracellular amino-terminus of the cognate receptors for polypeptide ligands, prior to a conformational change drawing the peptide toward the transmembrane helices [53]. Gelling et al. demonstrated the importance of the N-terminus of the GIP receptor for ligand binding and activation [54]. Given that GIP_{1–14} and GIP_{19–30} both demonstrate receptor-binding ability (Fig. 1 and Table 1), and that the high-affinity binding domain of GIP resides within residues 6–30 [12], it is likely that multiple contact residues contribute to high-affinity receptor binding. Furthermore, the body of evidence demonstrating the importance of the two N-terminal residues of GIP [9,10] combined with the bioactivity of GIP_{19–30} suggest an interaction or close proximity of the amino-terminus of GIP and its core region (possibly indicating the presence of a functional hinge in the α -helices), resulting in receptor activation. Until the solution structure for GIP is known and/or the contact residues are established by photo-affinity cross-linking, it is not possible to extend this mechanistic hypothesis without extensive testing of further GIP analogs.

The current work has verified the importance of the amino-terminus of GIP for bioactivity. GIP_{1–14} is a unique GIP fragment that displays specific GIP-receptor binding, and cAMP production in transfected cells, in addition to insulinotropic activity in the perfused pancreas and improvement of glucose tolerance in vivo. Consistent with prior studies in the perfused pancreas, synthetic GIP_{19–30} was able to weakly stimulate cAMP production and had intrinsic receptor binding ability. Hence, it appears that the two insulinotropic domains exhibit secondary structure within GIP_{1–14} and GIP_{19–30}, while a putative enterogastrone/somatostatinotropic domain lies in the C-terminus of the molecule.

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