Sequence Analysis of a Growth Hormone Releasing Factor from a Human Pancreatic Islet Tumor†

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ABSTRACT: A growth hormone releasing factor of a human pancreatic islet tumor (hpGRF) of an acromegalic patient was purified and subjected to Edman degradation in a spinning cup sequencer. Approximately 0.7–1.2 nmol of peptide was applied to the cup without any pretreatment, after coupling to 3-sulfophenyl isothiocyanate or after cleavage with cyanogen bromide, staphylococcal protease, or trypsin. On the basis of the analytical data, the N-terminal sequence of 39 residues is established to be H-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-. It is proposed that alanine is residue 40 and represents (as free acid) the C terminus of hpGRF. Synthetic hpGRF(1–40)-OH is highly potent in stimulating GH secretion from the rat anterior pituitary in vitro and in vivo. The C-terminal sequence of hpGRF does not appear to contribute significantly to the biologic intrinsic activity and potency of hpGRF, as demonstrated by the fact that the natural product and the synthetic peptides hpGRF(1–40)-OH, hpGRF(1–40)-NH_2, and hpGRF(1–29)-NH_2 show equivalent in vitro activities. On the basis of sequence homologies, hpGRF is closely related to members of the glucagon secretin family, especially to the porcine gut peptide PHI.

More than 30 years ago, Green & Harris (1949) postulated that the secretion of the anterior pituitary hormones is under the control of hypothalamic factors reaching the anterior pituitary through the hypophyseal portal vessel system. Since then, chemical characterization of several hypothalamic hypophysiotropic peptides, thyrotropin-releasing hormone (TRH) (Burgus et al., 1970; Nair et al., 1970), luteinizing hormone-releasing hormone (LH-RH) (Matsuo et al., 1971; Burgus et al., 1972), growth hormone release inhibiting factor, somatostatin (Brazeau et al., 1973; Burgus et al., 1973), and recently corticotropin-releasing factor (CRF) (Vale et al., 1981; Spiess et al., 1981a), has been accomplished.

Growth hormone (GH) releasing activity has been detected in extracts of hypothalamus [see Buckingham (1978) for a review] and certain human carcinoid and pancreatic islet tumors (Zafar et al., 1979; Frohman & Szabo, 1981; Leveston et al., 1981). It is widely accepted that this GH releasing activity is exhibited by a growth hormone releasing factor (GRF) that is involved together with somatostatin in the hypothalamic regulation of GH secretion from the anterior pituitary (Reichlin, 1961; Martin et al., 1978). Although several peptides with proposed GH releasing activity have been characterized (Schally et al., 1971; Yudaev et al., 1973; Villarreal et al., 1976), none is of high potency and considered likely to be of physiologic significance (Buckingham, 1978).

Recently, Thorner et al. (1982) described a 21-year-old woman with acromegaly and elevated GH plasma levels that abated immediately following removal of a pancreatic islet tumor. We (Thorner et al., 1982) and others (Böhlen et al., 1982) ascertained the presence of GH releasing activity in the tumor extract and undertook the purification (J. Rivier, J. Spiess, M. Thorner, and W. Vale, unpublished results) and characterization of this human pancreatic islet tumor GRF (hpGRF).

Experimental Procedures

Materials. Peptides were synthesized by using the solid-phase approaches described earlier (Rivier, 1974; Märki et al., 1981). Bovine L-1-(tosylamido)-2-phenylethyl chloromethyl ketone–trypsin (TPCK–trypsin) was purchased from Worthington and staphylococcal protease was from Miles. CNBr was obtained from Eastman. 3-Sulfophenyl isothiocyanate (3-SPITC) was purchased from Pierce and used without further purification. All organic solvents were redistilled.

Amino Acid Analysis. Amino acid analysis was performed with a Beckman 121 MB amino acid analyzer. The coefficient of variation was usually between 1% and 10%. More details are given elsewhere (Spiess et al., 1979, 1981b).

Edman Degradation. Peptides (0.7–1.5 nmol) were subjected to Edman degradation in a Wittmann-Liebold (1980) modified Beckman 890C spinning cup sequencer equipped with a variable speed drive (1000–6000 rpm), automatic converting flask, and a microprocessor manufactured by Horst Graffunder, Max Planck Institute for Molecular Genetics, West Berlin. Phenylthiohydantoin (PTH)-amino acids were determined with reverse-phase high-pressure liquid chromatography (HPLC). The coefficient of variation was 1–6%. The analytical procedure was similar to the one described (Spiess et al., 1982). Repetitive yields of stable PTH-amino acids were greater than 95%. Carry-over was usually smaller than 10%.

CNBr Cleavage. Approximately 0.7–1.0 nmol of peptide was incubated for 24 h at 22 °C under argon atmosphere in the presence of 50 pmol of bovine serum albumin (Pentex) with 30 μL of a freshly prepared solution of 20 mg of CNBr/mL of 75% aqueous formic acid. The sample was dried, dissolved in 50 μL of 0.33 M Quadrol (pH 9.2), and applied to the sequencer. The small amount of albumin (added to prevent peptide losses during the long incubation period) did not interfere in the analysis, as demonstrated in experiments with synthetic CRF.

Cleavage with Staphylococcal Protease. Peptide (0.7 nmol) was digested at 37 °C for 6 h under argon atmosphere with staphylococcal protease (enzyme:substrate weight ratio 1:5)

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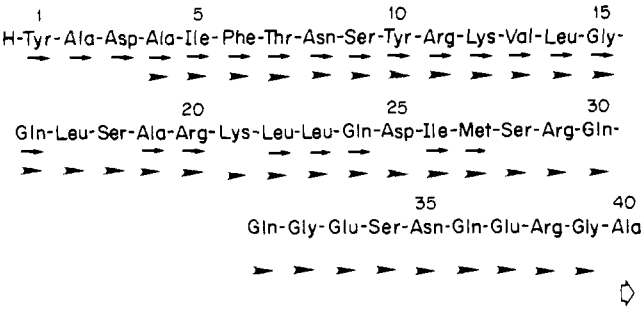

FIGURE 1: Amino acid sequence of human pancreatic GRF: (→) residues identified by sequence analysis of nonmodified hpGRF; (solid arrowhead) sequence established after coupling to 3-SPITC; (open arrowhead) residue suggested on the basis of sequence analysis of the tryptic hpGRF fragments and HPLC and tritiation data.

in 0.05 M Quadrol–1 mM EDTA, adjusted to pH 7.8 with trifluoroacetic acid. The sample was then applied to the spinning cup.

Cleavage with Trypsin. Peptide (0.7–1.2 nmol) was acetylated with acetic anhydride in aqueous pyridine. The product was digested with TPCK–trypsin (enzyme:substrate weight ratio 1:7) for 4.5 h at 37 °C in 0.05 M Quadrol–5 mM $CaCl_2$ (pH 8.0) and applied to the cup.

Bioassay. Growth hormone releasing activity was determined by the ability to stimulate the secretion of growth hormone from rat anterior pituitary cells in monolayer culture (Vale et al., 1975). Sensitivity to GRF in this assay was greatly enhanced by addition of 5 nM dexamethasone and 30 pM triiodothyronine to the culture medium (W. Vale, C. Rivier, J. Spiess, J. Vaughan, G. Yamamoto, and J. Rivier, unpublished results).

Results

The acidic extract of a human GRF producing pancreatic tumor (wet weight 5 g) was subjected to gel filtration (Sephadex G-50) and reverse-phase HPLC on Vydac diphenyl and C_{18} columns to yield 33 μg of peptide, as determined by amino acid analysis. This amount corresponded to 5–6 nmol of peptide based on the assumption that hpGRF contained four arginine residues per molecule (see below). Under the conditions of reverse-phase HPLC used, no contamination of the purified hpGRF with other peptides could be detected.

Approximately 6.5 μg (corresponding to 1.1 nmol) of this purified hpGRF was subjected to Edman degradation in the spinning cup. In every degradation cycle, only one major PTH-amino acid was identified with reverse-phase HPLC. Assignment of these major PTH-amino acids to one peptide (hpGRF) was justified by the observation that the relative PTH-amino acid yields of two consecutive degradation cycles were in agreement with the corresponding ratios obtained in sequencer experiments with synthetic peptides. The N-terminal 27 residues with the exception of four residues were determined (Figure 1). The results of this experiment were confirmed and supplemented by sequence analysis of 5.9 μg (1.0 nmol) of natural hpGRF, coupled to 3-sulfophenyl isothiocyanate to improve the binding of the peptide to the positively charged Polybrene film in the spinning cup. Residues 4–39 of hpGRF were identified in this experiment (Figure 2). All PTH-amino acids were quantified with the exception of the PTH derivative of 3-sulfophenylthiocarbamyllysine (PTH-N^{ϵ}-SPTC-lysine), which was only qualitatively analyzed with reverse-phase HPLC. The N-terminal three residues could not be detected with reverse-phase HPLC because of interfering contaminants. On the basis of PTH-amino acid yields, hpGRF represented approximately 75–85% of the peptide degraded. A similar range was accordingly assumed for the purity of hpGRF in the analyzed fraction.

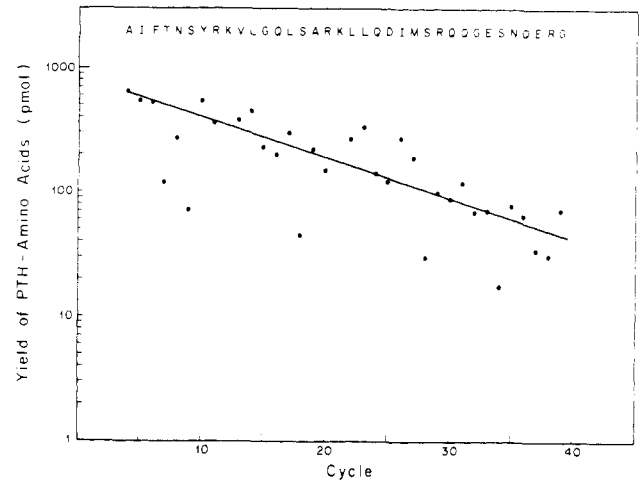

FIGURE 2: PTH-amino acid yields from automated Edman degradation of human pancreatic GRF coupled to 3-SPITC. Peptide (5.9 μg, 1.0 nmol) was incubated for 80 min at 42 °C in 40 mM 3-SPITC and 0.33 M Quadrol buffer (pH 9.2). A second coupling was performed in the cup with phenyl isothiocyanate. The cleavage with heptafluorobutyric acid was repeated in the first cycle. PTH-N^{ϵ}-SPTC-lysine was identified but no quantified. Total PTH-amino acid yields are presented.

The primary structure of hpGRF, especially its C-terminal sequence, was further investigated by Edman degradation of fragments cleaved from hpGRF with cyanogen bromide, trypsin, or staphylococcal protease. The mixture of hpGRF fragments was subjected to sequence analysis without purification to avoid losses.

The finding that cyanogen bromide cleavage of 4.1 μg (0.7 nmol) of peptide yielded two fragments, identified by Edman degradation as hpGRF(1–27) and a C-terminal peptide, indicated the presence of only one methionine residue (methionine-27) in hpGRF, which was recognized in the sequence analysis of the noncleaved peptide (Figures 1 and 2).

When hpGRF was acetylated at its lysine residues and the N terminus, selectively cleaved with trypsin at its arginine residues, and degraded in the spinning cup, most of the PTH-amino acids, determined in every cycle, could be assigned to one of four hpGRF fragments (Table I). The conclusion, that hpGRF contained four arginine residues, was in agreement with the data obtained by sequence analysis of hpGRF derivatized with 3-SPITC (Figures 1 and 2).

It was observed that in the first cycle the yield of PTH-glycine, assigned to glycine-39 of hpGRF, was smaller than the yields of the other PTH-amino acids of the same cycle. It was improbable that this finding had to be referred to incomplete tryptic cleavage, the conditions of which had been worked out in kinetic experiments with hpGRF analogues digested and subsequently analyzed with the sequencer. It was therefore assumed that the relatively low yield of PTH-glycine in cycle 1 meant that glycine-39 was the N terminus of a short C-terminal tryptic fragment of few residues. Analysis of the relative PTH-amino acid yields of cycles 2 and 3 (Table I) justified the tentative assignment of alanine and phenylalanine to the C-terminal fragment.

This interpretation of the role of glycine-39 was supported by the data of sequence analysis of hpGRF digested with staphylococcal protease, which is known to cleave peptides at the C-terminal side of glutamic acid and, to a lesser extent, aspartic acid residues (Houmard & Drapeau, 1972). Digestion

Table I: Edman Degradation of Tryptic hpGRF Fragments[a]

assignment[b] of PTH-amino acids	cycle 1	2	3	4	5	6	7	8	9
GRF(12–20)	Lys	Val	Leu	Gly	Gln	Leu	Ser	Ala	Arg
yield (pmol)	899[c]	384	914[c]	281	224	381	17	188	150[d]
GRF(21–29)	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg
yield (pmol)	899[c]	536	914[c]	217	151	428	269	23	150[d]
GRF(30–38)	Gln	Gln	Gly	Glu	Ser	Asn	Gln	Glu	Arg
yield (pmol)	263	302	258	313	26	132	154	85	150[d]
Gly-39	Gly								
yield (pmol)	138								
nonassigned PTH-amino acids		Ala	Phe			Lys/Val		Leu	
yield (pmol)		181	116			28/42		51	

[a] hpGRF (6.9 μg, 1.2 nmol) was acetylated, digested with trypsin, and degraded in the spinning cup. [b] PTH-amino acids determined in different cycles were assigned to hpGRF fragments. Total PTH-amino acid yields are presented. [c] Yield has to be referred to two residues of hpGRF. [d] Yield has to be referred to three residues of hpGRF.

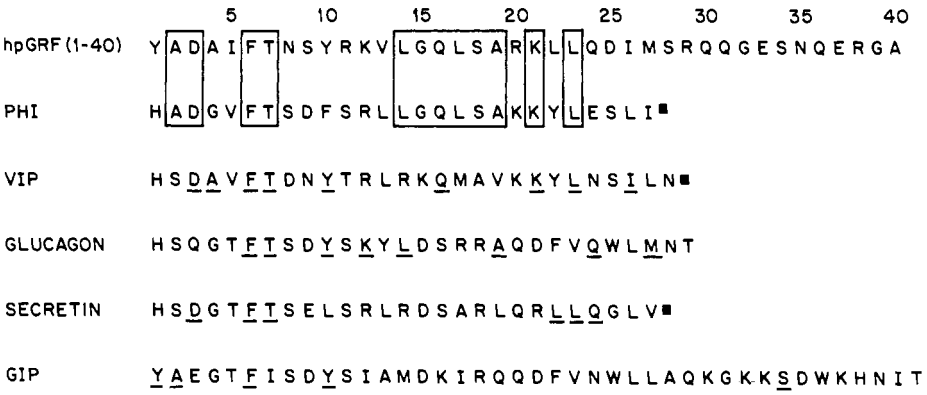

FIGURE 3: Homologies between hpGRF(1–40) and peptides of the glucagon secretin family (GIP, gastric inhibitory peptide; PHI, peptide with histidine as N terminus and isoleucine amide as C terminus; VIP, vasoactive intestinal peptide). Alignments of hpGRF with PHI are marked by rectangles. Alignments of other peptides with hpGRF are underlined. (■) Amidated C terminus.

of 4.1 μg (0.7 nmol) of hpGRF yielded three fragments, hpGRF(1–33), hpGRF(34–37), and a C-terminal fragment starting with arginine-38. The yields of PTH-arginine (cycle 1, assigned to arginine-38) and PTH-glycine (cycle 2, assigned to glycine-39) were 300 and 120 pmol, respectively. The difference between these yields was suggestive for a rapid disappearance of the C-terminal hpGRF fragment. In cycle 3, evidence for alanine was provided, suggesting that hpGRF residue 40 was alanine.

In view of the sequence analyses of hpGRF and its fragments, it was hypothesized that residue 40 (alanine or alanine amide) was the C terminus of hpGRF. Amino acid analysis of natural hpGRF revealed that the molar amino acid ratios, normalized to four arginine residues or one methionine residue, were not in complete agreement with the composition of hpGRF(1–40) (Table II). However, in reverse-phase HPLC on a Vydac C_{18} column, an aliquot of further purified hpGRF (20 of 220 pmol) coeluted with synthetic hpGRF(1–40)-OH under highly resolving conditions (Rivier et al., unpublished results). When another aliquot (180 pmol) of the same fraction was C terminally tritiated as described (Spiess et al., 1981a), tritium was predominantly incorporated into alanine (840 cpm/nmol). These data of HPLC and tritiation supported the conclusion that hpGRF was identical with hpGRF(1–40)-OH.

It was in agreement with this conclusion that natural hpGRF and synthetic hpGRF(1–40)-OH did not differ significantly in their potencies to stimulate GH secretion in vitro. The minimally and half-maximally effective concentrations of hpGRF(1–40)-OH were 3–8 and 20–100 pM, respectively. At maximally effective peptide concentrations (>1 nM), GH secretory rates were elevated 8–20-fold (Rivier et al., unpublished results). The intravenous administration of synthetic

Table II: Amino Acid Composition of Human Pancreatic GRF

amino acid	acid hydrolysis[a]	sequence analysis[b]	amino acid	acid hydrolysis[a]	sequence analysis[b]
Asx	4.7[c] (5)	4	Met	0.9 (1)	1
Thr	2.1 (2)	1	Ile	2.4 (2)	2
Ser	3.8 (4)	4	Leu	4.6 (5)	4
Glx	7.1 (7)	7	Tyr	1.7 (2)	2
Pro	1.3 (1)	0	Phe	1.5 (2)	1
Gly	3.3 (3)	3	Lys	3.2 (3)	2
Ala	3.5 (4)	4	His	0.4 (0)	0
Cys	0 (0)	0	Trp	0 (0)	0
Val	1.9 (2)	1	Arg	4.0 (4)	4

[a] GRF (2.2 μg) was hydrolyzed for 22 h at 110 °C with 4 M methanesulfonic acid and 0.2% tryptamine. Norleucine was added as an internal standard. [b] The composition of hpGRF(1–40) is presented. [c] Amino acid ratios are presented; the nearest integer is given in parentheses. Values were not corrected for losses during hydrolysis with the exception of the serine value.

hpGRF(1–40)-OH or hpGRF(1–40)-NH_2 (40 ng to 25 μg of peptide/kg of body weight) increased GH plasma levels in the rat by more than 10-fold at highest doses (C. Rivier, J. Rivier, J. Spiess, and W. Vale, unpublished results). In these experiments, spontaneous GH secretion was suppressed by pretreatment with a dopamine β-hydroxylase inhibitor (Cass & Martin, 1981).

Not only synthetic peptides such as hpGRF(1–40) and hpGRF(1–39) with free or amidated C termini but also shorter fragments including hpGRF(1–29)-NH_2 possessed the full biologic intrinsic activity (ability to elicit a maximal response at highest concentration) and potency of natural hpGRF in the in vitro assay.

Discussion

By microsequence analysis of hpGRF and its fragments in the spinning cup, the sequence of the N-terminal 39 residues has been unambiguously established. The relatively high yields of the PTH derivative of glycine-39, which were obtained in two different sequence analyses of the proteolytic hpGRF fragments, indicate in our experience that glycine-39 is unlikely to be the C terminus of hpGRF. It is therefore probable that hpGRF contains 40 or more residues. On the basis of the sequence analysis of the proteolytic fragments, alanine is assumed as residue 40. In view of the rapid disappearance of the C-terminal hpGRF fragments from the spinning cup, the behavior of the 3-SPITC-treated peptides (Figure 2), and the data of reverse-phase HPLC and C-terminal tritiation, it is proposed that residue 40 (as free acid) represents the C terminus of hpGRF. The discrepancy between the amino acid compositions of the hpGRF fraction and hpGRF(1–40) could reflect the presence of other peptides (related or unrelated to hpGRF) in the analyzed fraction.

The finding that the C-terminal sequence does not seem to contribute significantly to the intrinsic activity and potency of hpGRF, as demonstrated by in vitro bioassays with synthetic hpGRF fragments, contrasts with the observation of the essential role of the C-terminal sequence for intrinsic activity and potency in the case of the known hypothalamic hypophysiotropic peptides CRF, LH-RH, and TRH (Vale et al., 1977, 1981).

It is interesting that hpGRF belongs to the glucagon secretin family and is especially closely related to the 27 residue peptide PHI, isolated from porcine gut (Tatemoto & Mutt, 1981) (Figure 3). PHI and hpGRF align with 12 residues. Twelve residues of the remaining 15 residues of PHI could result from single base changes. Despite this homology, PHI or [Gln^{24}]PHI (or VIP or glucagon) possesses less than 0.01% of the potency of hpGRF(1–40) in stimulating GH secretion.

The structural relationship between hpGRF and mammalian hypothalamic GRF has been investigated by sequence analysis of purified rat hypothalamic GRF. Initial analyses are suggestive for significant homologies between the N-terminal sequences of hpGRF and hypothalamic GRF. It is therefore anticipated that hpGRF(1–40)-OH is closely related to the long sought for GH releasing factor.

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