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SYNTHESIS AND IN VITRO BIOACTIVITY OF HUMAN GROWTH HORMONE-RELEASING FACTOR ANALOGS SUBSTITUTED AT POSITION-1*

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SUMMARY. Eight position-1 analogs of the 40-amino acid fragment and two position-1 analogs of human growth hormone-releasing factor were synthesized by solid phase methodology and their capacity to release growth hormone was determined using rat anterior pituitary cells in monolayer culture. Relative to hGRF(1-40)OH, which was arbitrarily assigned a potency value of 1, [D-Tyr^1]hGRF(1-40)OH, [Phe^1]hGRF(1-40)OH, [Trp^1]hGRF(1-40)OH, [His^1]hGRF(1-40)OH, [Ala^1]hGRF(1-40)OH, [(N-Ac)Tyr^1]hGRF(1-40)OH, Arg^0-hGRF(1-40)OH and Ala^0-hGRF(1-40)OH have potencies of 0.022, 0.038, 0.003, 0.351, 0.010, 0.032, 0.002 and 0.007 respectively. Relative to hGRF(1-44)NH_2 = 1, [(3-Me)His^1]hGRF(1-44)NH_2 and [[O-Me)Tyr^1]hGRF(1-44)NH_2 have potiencies of 0.132 and 0.001 respectively. These results demonstrate the prerequisite for an aromatic residue at position-1 for potent biological activity and also suggest that the capacity for hydrogen bond formation with the first residue is required for full receptor-ligand interaction.

Recently, we reported the isolation and characterization of three growth hormone-releasing peptides from a tumor of the pancreas (hpGRF) obtained from an acromegalic patient in Lyon, France (1). The most potent hpGRF in a pituitary monolayer culture assay contains forty-four amino acids (hpGRF(1-44)NH_2) and its primary structure is: 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-Ala-Arg-Ala-Arg-Leu-NH_2. The other two hpGRF peptides correspond to the NH_2-terminal forty (hpGRF(1-40)OH)

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ABBREVIATIONS: hpGRF(1-44)NH_2, hpGRF(1-40)OH and hpGRF(1-37)OH are the 44-, 40- and 37-amino acid forms of human pancreas growth hormone-releasing factor, respectively. hGRF designates human growth hormone-releasing factor, tumorous or hypothalamic. HPLC = high performance liquid chromatography.

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and thirty-seven (hpGRF(1-37)OH) amino acids of hpGRF(1-44)NH_2 with their COOH-termini as free carboxylic acids. In this tumor, hpGRF(1-40)OH was present in the highest concentration while hpGRF(1-37)OH was the lowest (1). From another acromegalic patient in Charlottesville, USA, a second pancreatic tumor was obtained which, upon extraction and purification, yielded only the same growth hormone-releasing peptide as hpGRF(1-40)OH (2,3). More recently, the primary structure of human hypothalamic GRF has been characterized as being identical to hpGRF(1-44)NH_2 (9) and thus we shall only use the term hGRF to denote the human growth hormone releasing factor, tumorous or hypothalamic.

In the original paper reporting the isolation of the three hGRF peptides, we had noted that the tyrosine in position-1 is required for the high *in vitro* bioactivity of hGRF because deletion of that residue will reduce the activity of the resulting des-Tyr^1-analog to 0.1% of that of the parent molecule (1). To further delineate the importance of the Tyr^1-residue we have synthesized eight position-1 modified analogs of hGRF(1-40)OH and two analogs of hGRF(1-44)NH_2 substituted at the Tyr^1-amino acid and determined their growth hormone-releasing activity in the rat anterior pituitary cell culture assay (4). The *in vitro* bioassay results of these analogs are reported here.

MATERIALS AND METHODS

Derivatized amino acids used in the synthesis reported here were of the L-configuration unless stated otherwise and purchased from Bachem, Inc. The N^{α}-amino function was protected exclusively with the t-butyloxycarbonyl (Boc) group. Side chain functional groups were protected as follows: benzyl for threonine, serine, D-tyrosine, glutamic and aspartic acids; 2,6-dichlorobenzyl for tyrosine; p-toluenesulfonyl for histidine and arginine; 2-chlorobenzyloxy-carbonyl for lysine. Chloromethylated polystyrene resin cross-linked with 1% divinylbenzene was obtained from Lab Systems, Inc. The substitution was 0.9 mmol chlorine per gram resin according to the manufacturer. p-Methylbenzhyl-drylamine resin was synthesized in our laboratory using a previously published procedure (5). The substitution was 0.60 mmol amine per gram by Gisin analysis (6). Amino acid analyses were determined on peptide hydrolysates using a Kontron Liquimat III amino acid analyzer equipped with a post-column o-phthalaldehyde derivatization detection system (7). Hydrolyses were performed in 6 N HCl containing 2.5% thioglycollic acid at 110° for 20 hours in tubes sealed under vacuum. Optical rotations were measured at c=1 in 1% AcOH solution at room temperature with a Perkin-Elmer model 141 polarimeter. Reverse-phase high performance liquid chromatography (HPLC) was performed on a Waters Associate model 204 liquid chromatography system using a 5 μ particle size, 0.46 X 25 cm Altex Ultrasphere-ODS column and a solvent system composed of buffer A: 0.25 N triethylammonium phosphate at pH 3.00 and buffer B: 20% buffer A in acetonitrile by volume. The column was eluted at a flow rate of

1.5 ml/min and the column effluent detected by UV absorption at 210 nm with 0.1 absorbance unit at full scale and a recorder chart speed of 30 cm/h. Additional HPLC conditions are given in legends to Tables 3 and 4.

Peptide Synthesis: The position-1 modified analogs of hGRF were synthesized by solid phase methodology (8) on a Beckman Model 990 peptide synthesizer according to the procedure used in the synthesis of human hypothalamic GRF (9). For preparing the hGRF(1-40)OH analogs, Boc-Ala-OCH_2-polystyrene resin substituted with 0.56 mmol alanine per gram resin was prepared by coupling Boc-Ala to the chloromethylated polystyrene resin according to a previously published method (10). Starting with 3.00 g of the Boc-Ala-resin, each of the derivatized amino acids corresponding to the sequence of hGRF was coupled successively on the resin to yield 8.80 g protected hGRF(2-40)-OCH_2-polystyrene resin. Aliquots of 1 g were removed and used to prepare the respective hGRF(1-40)OH analogs modified at position-1. [(N-Ac)Tyr^1]hGRF(1-40)OH was prepared by acetylation of the N^{α}-amino deblocked hGRF(1-40)-OCH_2-polystyrene resin with acetic anhydride (9). For preparing the hGRF(1-44)NH_2 analogs, 6.00 g of the p-methylbenzhydrylamine resin was coupled successively with each of the derivatized amino acids corresponding to the sequence of hGRF to yield 16.11 g protected hGRF(2-44)-NH-CH($C_6H_5CH_3$)-polystyrene resin. Aliquots of 1 g were removed and used to prepare the respective Tyr^1-modified hGRF(1-44)NH_2 analogs.

After completion of the synthesis, the protected peptide-resin was treated with a mixture consisting of 1.5 ml anisole, 0.25 ml methylethylsulfide and 10 ml hydrogen fluoride per gram of peptide-resin at -20°C for one-half hour and at 0°C for one-half hour to cleave the peptide from the resin anchor as well as to deprotect the side chain functional groups. The resulting crude peptide product was purified by gel filtration on Sephadex G-50 fine (Pharmacia), cation-exchange chromatography (Whatman CM-32 cellulose) and partition chromatography on Sephadex G-50 fine. Details of the purification method have been published (9). The purified peptides were characterized by amino acid analysis, optical rotation and high performance liquid chromatography.

Bioassays: The capacity of the position-1 modified analogs of hGRF to release growth hormone was tested in four-day old rat anterior pituitary cells as described (4). Graded doses of the peptides were incubated in triplicate for four hours with the cells and the amount of growth hormone released into the culture medium at the end of the incubation period was determined by a radioimmunoassay using a monkey anti-mouse growth hormone serum kindley provided by Dr. Y. Sinha (11) and a rat growth hormone preparation (RP-1) generously furnished by NIADDKD of NIH. Potency estimates of the various analogs were calculated using the computer program BIOPROG (12).

RESULTS AND DISCUSSION

Eight hGRF(1-40)OH analogs and two hGRF(1-44)NH_2 analogs substituted at position-1 were synthesized by solid phase methodology. All of these synthetic peptides yielded the correct amino acid composition after HCl hydrolysis as shown in tables 1 and 2 and their purity is greater than 95% by HPLC analysis. The major contaminant is the corresponding methionine sulfoxide analog of the parent compound. The physical properties of these synthetic peptides are presented in tables 3 and 4.

TABLE 1. AMINO ACID COMPOSITION OF hGRF(1-40)OH ANALOGS MODIFIED AT POSITION-1

	[D-Tyr^1]	[Phe^1]	[Trp^1]	[His^1]	[Ala^1]	(N-Ac)Tyr^1]	Arg^6-	Ala^8-
Asx	4.18 (4)*	4.17 (4)	4.12 (4)	4.03 (4)	4.11 (4)	4.00 (1)	3.99 (4)	4.09 (4)
Thr	0.83 (1)	1.08 (1)	1.08 (1)	1.05 (1)	0.89 (1)	1.04 (1)	1.08 (1)	1.05 (1)
Ser	4.01 (4)	3.89 (4)	3.88 (4)	3.89 (4)	3.63 (4)	3.81 (4)	3.93 (4)	3.89 (4)
Glx	7.20 (7)	7.53 (7)	7.40 (7)	7.33 (7)	7.48 (7)	7.31 (7)	7.28 (7)	7.44 (7)
Gly	2.92 (3)	3.08 (3)	3.00 (3)	3.00 (3)	2.91 (3)	2.97 (3)	3.05 (3)	3.03 (3)
Ala	3.93 (4)	3.92 (4)	3.81 (4)	3.82 (4)	5.12 (5)	3.85 (4)	3.82 (4)	4.90 (5)
Val	0.80 (1)	0.86 (1)	0.93 (1)	0.90 (1)	0.86 (1)	0.84 (1)	0.92 (1)	0.81 (1)
Met	1.28 (1)	0.77 (1)	0.97 (1)	1.10 (1)	0.94 (1)	0.80 (1)	1.24 (1)	0.86 (1)
Ile	1.99 (2)	1.99 (2)	2.02 (2)	2.03 (2)	1.87 (2)	1.99 (2)	2.05 (2)	2.00 (2)
Leu	4.16 (4)	4.09 (4)	4.05 (4)	4.13 (4)	4.06 (4)	4.06 (4)	4.10 (4)	4.17 (4)
Tyr	2.12 (2)	1.04 (1)	1.04 (1)	1.03 (1)	0.95 (1)	2.26 (2)	1.97 (2)	2.13 (2)
Phe	0.83 (1)	1.95 (2)	0.92 (1)	0.92 (1)	1.12 (1)	1.30 (1)	0.94 (1)	0.89 (1)
His	-	-	-	1.02 (1)	-	-	-	-
Trp	-	-	1.14 (1)	-	-	-	-	-
Lys	1.82 (2)	1.82 (2)	1.81 (2)	1.96 (2)	1.86 (2)	1.84 (2)	1.89 (2)	1.86 (2)
Arg	3.91 (4)	3.82 (4)	3.84 (4)	3.81 (4)	4.21 (4)	3.94 (4)	4.75 (5)	3.88 (4)

* The numbers within the brackets represent the integer amino acid composition of the synthetic peptides.

When the position-1 modified analogs of hGRF(1-40)OH were tested for their capacity to release growth hormone on a rat anterior pituitary monolayer culture system, only the [His^1]-substituted compound showed substantial bioactivity as compared to the parent hGRF(1-40)OH molecule (see Table 5). This finding is not surprising since rat GRF has histidine as the first NH_2-terminal amino acid (13). In spite of the potency differences, all eight

TABLE 2. AMINO ACID COMPOSITION OF hGRF(1-44)NH_2 ANALOGS MODIFIED AT POSITION-1

	[(3-Me)His^1]	[(O-Me)Tyr^1]+
Asx	4.08 (4)*	4.22 (4)
Thr	0.86 (1)	0.81 (1)
Ser	3.59 (4)	3.13 (4)
Glx	7.48 (7)	7.74 (7)
Gly	2.96 (3)	2.97 (3)
Ala	5.18 (5)	4.99 (5)
Val	0.94 (1)	1.05 (1)
Met	0.98 (1)	0.95 (1)
Ile	1.83 (2)	2.00 (2)
Leu	5.19 (5)	5.43 (5)
Tyr	1.08 (1)	1.81 (2)
Phe	0.87 (1)	0.94 (1)
(3-Me)His	0.80 (1)	-
Lys	1.91 (2)	1.89 (2)
Arg	6.25 (6)	6.07 (6)

*The numbers within the brackets represent the integer amino acid composition of the synthetic peptides.
+[(O-Me)Tyr^1]hGRF(1-44)NH_2 was hydrolyzed for 72 hour before analysis.

TABLE 3. PHYSICAL PROPERTIES OF hGRF(1-40)OH ANALOGS MODIFIED AT POSITION-1

	[D-Tyr^1]	[Phe^1]	[Trp^1]	[His^1]	[Ala^1]	[(N-Ac)Tyr^1]	Arg^0-	Ala^0-
$[\alpha]_D$	-68.8±1.0	-64.8±1.0	-63.0±1.0	-67.3±1.0	-66.4±1.0	-61.3±1.0	-61.9±1.0	-61.0±1.0
HPLC* Retention Time	40.68 min	37.23 min	43.14 min	28.57 min	28.14 min	52.66 min	34.86 min	36.46 min

*HPLC was performed as described in Materials and Methods. All of the peptides except [Trp^1]hGRF(1-40)OH were eluted with a linear gradient of 35% to 40% buffer B added to buffer A in 50 min, followed by 40% buffer B in buffer A isocratically until the peptides eluted from the column. The [Trp^1]-analog was eluted by a linear gradient of 37% to 40% buffer B added to buffer A in 60 min.

position-1 analogs gave parallel dose-response curves and reached the same maximal stimulation of growth hormone release as hGRF(1-40)OH (data not shown).

Substitution with other aromatic residues such as phenylalanine or tryptophan resulted in much reduced bioactivity, especially for [Tyr]^1-hGRF(1-40)OH. To determine whether the aromatic side chain function is required for imparting the molecule with high bioactivity, [Ala^1]hGRF(1-40)OH was prepared and tested. Results showed that the [Ala^1]-substituted analog retained only 1% the activity of hGRF(1-40)OH. Interestingly, replacement of the NH_2-terminal tyrosine with its D-isomer resulted in a 50-fold decrease of activity when compared to the parent molecule, indicating that preserving the native peptide-backbone conformation is important for binding and activating the receptor.

Blocking of the NH_2-terminal of hGRF with an acetyl group reduced its activity to 3% of that of the parent compound. However, the reduced activity may be caused by steric hindrance rather than by elimination of the N^{α}-amino

TABLE 4. PHYSICAL PROPERTIES OF hGRF(1-44)NH_2 ANALOGS MODIFIED AT POSITION-1

	[(3-Me)His^1]	[(O-Me)Tyr^1]
$[\alpha]_D$	-63.3 ± 1.0	-59.9 ± 1.0
HPLC* Retention Time	35.60 min	46.40 min

*HPLC was performed as described in Materials and Methods. All of the peptides were eluted with a linear gradient of 30% to 40% buffer B added to buffer A in 60 min.

TABLE 5. RELATIVE POTENCIES OF hGRF(1-40)OH ANALOGS MODIFIED AT POSITION-1

Analog	Potency*	95% Confidence limits
[D-Tyr^1]hGRF(1-40)OH	0.022	0.015-0.036
[Phe^1]hGRF(1-40)OH	0.038	0.031-0.048
[Trp^1]hGRF(1-40)OH	0.003	0.002-0.005
[His^1]hGRF(1-40)OH	0.351	0.254-0.478
[Ala^1]hGRF(1-40)OH	0.010	0.008-0.014
[(N-Ac)Tyr^1]hGRF(1-40)OH	0.032	0.021-0.052
Arg^0-hGRF(1-40)OH	0.002	0.001-0.002
Ala^0-hGRF(1-40)OH	0.007	0.005-0.010

*hGRF(1-40)OH potency = 1

function since Ala^0-hGRF(1-40)OH which contains an NH_2-terminal amino function was much less active than [(N-Ac)Tyr^1]hGRF(1-40)OH. Enhancing the positively charged characteristic at the NH_2-terminal of hGRF did not lead to an increase in potency because the analog, Arg^0-hGRF(1-40)OH, was even less active than Ala^0-hGRF(1-40)OH.

Both the phenolic hydroxyl group and the imidazole amino group in the Tyr^1- and His^1-side chains, respectively, have the ability to form hydrogen bonding with other functional group on the peptide or the receptor. To test the importance of this interaction in the receptor-ligand reaction for GRF, the [(O-Me)Tyr^1]- and [(3-Me)His^1]-analog of hGRF(1-44)NH_2 were synthesized and bioassayed. Results showed that methylation of the phenolic hydroxyl group of Tyr^1 greatly reduced its bioactivity as compared with the parent molecule (see table 6). However, the decrease in potency was not that pronounced when a 3-methyl group was added to the imidazole ring.

The bioassay results from this limited study thus show that the first NH_2-terminal residue of hGRF requires an aromatic amino acid that has the

TABLE 6. RELATIVE POTENCIES OF hGRF(1-44)NH_2 ANALONGS MODIFIED AT POSITION-1

Analog	Potency+	95% Confidence limits
[(3-Me)His^1]hGRF(1-44)NH_2	0.132	0.065-0.282
[(O-Me)Tyr^1]hGRF(1-44)NH_2	0.001	0.001-0.002

+hGRF(1-44)NH_2 potency = 1

potential to form hydrogen bonding with either the receptor or possibly another residue within hGRF. Moreover, the results indicate that the N^{α}-amino function at the NH_2-terminus may not be essential for imparting the molecule with high biological activity.

While this study was in progress, Lance et al (14). reported that [D-Tyr^1]hGRF(1-29)NH_2 and [(N-Ac)Tyr^1]hGRF(1-29)NH_2 were substantially more potent than the parent molecule hGRF(1-29)NH_2. These results are in disagreement with our present data. However, the bioassay used by Lance et al (14) to determine the potency of their analogs was an *in vivo* assay whereas ours was an *in vitro* system. In view of the prolonged stability of hGRF *in vitro* (unpublished data), the effects reported by Lance et al (14) may simply reflect a decreased enzymatic degradation of the NH_2-terminal analogs when administered *in vivo*.

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