J = 8.2 Hz, 2 H); MS (EI) m/e 392 (M^+), 201, 200, 188, 187 (100), 186, 119, 118, 91, 84, 70; IR ($CHCl_3$) ν (cm^{-1}) 1282, 1443, 1500, 1640, 2920.

5-[[5-[1-Hydroxy-3-[2-(4-methylphenyl)-5-methyl-4-oxazolyl]propyl]-2-thienyl]methyl]thiazolidine-2,4-dione (53) was prepared in 5 steps from **124** by the same sequence as that described for **119** and obtained as a white foam: ^1H NMR (300 MHz, $CDCl_3$) δ 2.13 (m, 2 H), 2.31 (s, 3 H), 2.38 (s, 3 H), 2.65 (m, 2 H), 3.39 (dd, J = 51.2, 8.8 Hz, 1 H), 3.59 (dd, J = 15.2, 3.8 Hz, 1 H), 4.50 (dd, J = 8.9, 3.7 Hz, 1 H), 5.01 (m, 1 H), 6.80 (m, 2 H), 7.24 (d, J = 7 Hz, 2 H), 7.88 (d, J = 6.6 Hz, 2 H); MS (EI) m/e 245, 201, 188, 187, 186, 141, 118; IR ($CHCl_3$) ν (cm^{-1}) 1700, 1750; HRMS calcd 428.0858, found 428.0749.

5-[[5-[3-[2-(4-Methylphenyl)-5-methyl-4-oxazolyl]-propionyl]-2-thienyl]methyl]thiazolidine-2,4-dione (57) was prepared by PDC oxidation of the alcohol **53**, as described above for **109** and obtained as a solid: mp 158-160 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 2.31 (s, 3 H), 2.36 (s, 3 H), 2.87 (t, J = 6.9 Hz, 2 H), 3.24 (t, J = 6.8 Hz, 2 H), 3.45 (dd, J = 15.1, 8.1 Hz, 1 H), 3.66 (dd, J = 14.9, 4 Hz, 1 H), 4.53 (dd, J = 8, 4 Hz, 1 H), 6.90 (d, J = 3.4 Hz, 1 H), 7.20 (d, J = 7.8 Hz, 2 H), 7.56 (d, J = 3.5 Hz, 1 H), 7.81 (d, J = 8.0 Hz, 2 H), 8.78 (br s, 1 H); MS (EI) m/e 440 (M^+), 200; IR (KBr) ν (cm^{-1}) 1670, 1700, 1750. Anal. ($C_{22}H_{20}N_2O_4S_2 \cdot {}^1/_2H_2O$) C, H, N.

5-[[5-[3-[2-(4-Methylphenyl)-5-methyl-4-oxazolyl]-1-propenyl]-2-thienyl]methyl]thiazolidine-2,4-dione (59). A solution of **105** (0.15 kg, 0.27 mol) in THF (600 mL) and 6 N HCl (600 mL) was stirred at room temperature for 45 min. The pH was adjusted to 5 with sodium bicarbonate and the solution was extracted with ethyl acetate (3×). The combined extracts were dried over magnesium sulfate and concentrated. The product was isolated by flash chromatography (30% ethyl acetate in hexanes) as an oil (42 g, 36%): ^1H NMR (300 MHz, $CDCl_3$) δ 2.31 (s, 3 H), 2.36 (s, 3 H), 3.28 (dd, J = 14.5, 8.8 Hz, 1 H), 3.36 (d, J = 6.7 Hz, 2 H), 3.55 (dd, J = 15.0, 3.8 Hz, 1 H), 4.45 (dd, J = 9.0, 3.8 Hz, 1 H), 6.11 (dt, J = 15.4, 7 Hz, 1 H), 6.47 (d, J = 15.4 Hz, 1 H), 6.69 (*AB*, J = 3.6 Hz, 1 H), 6.70 (*AB*, J = 3.7 Hz, 1 H), 7.20 (d, J = 7.9 Hz, 2 H), 7.84 (d, J = 8.2 Hz, 2 H), 9.0 (br s, 1 H); MS (EI) m/e 424 (M^+); IR ($CHCl_3$) ν (cm^{-1}) 1700. The product was converted to its sodium salt by the method described for **51**. Anal. ($C_{22}H_{19}N_2NaO_3S_2$) C, H, N.

5-[4-[3-[5-Methyl-2-(4-hydroxy-3,5-dimethylphenyl)-4-oxazolyl]propionyl]benzyl]thiazolidine-2,4-dione (45). A solution of **44** (105 mg, 0.21 mmol) in acetic acid (10 mL) and 48% HBr (5 mL) was heated to reflux for 1 h, cooled, poured into ice-water, and extracted with ethyl acetate. The combined extracts were washed with water, saturated sodium bicarbonate, water again, and brine, dried over magnesium sulfate, and concentrated (103 mg, 100%): ^1H NMR (300 MHz, $CDCl_3$) δ 2.22 (s, 6 H), 2.32 (s, 3 H), 2.86 (t, J = 7 Hz, 2 H), 3.15 (dd, J = 14, 9 Hz, 1 H), 3.30 (t, J = 7 Hz, 2 H), 3.47 (dd, J = 14, 4, Hz, 1 H), 4.49 (dd, J = 8, 4 Hz, 1 H), 7.23 (d, J = 8 Hz, 2 H), 7.56 (s, 2 H), 7.86 (d, J = 8.0 Hz, 2 H). The product was converted to its sodium salt as described for **26**: mp 230-240 °C. Anal. ($C_{25}H_{23}N_2NaO_5S \cdot 2H_2O$) C, H, N.

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Affinity of Human Growth Hormone-Releasing Factor (1-29)NH_2 Analogues for GRF Binding Sites in Rat Adenopituitary[1]

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Previous research on growth hormone-releasing factor analogues has used pituitary cell culture assay systems to evaluate in vitro their biological activity. However, binding assay systems in which receptor affinity and peptide stability can be assessed independently have been lacking so far. Since we have recently develop a sensitive GRF binding assay with [^{125}I-Tyr^{10}]hGRF(1-44)NH_2, this method was applied to structure-affinity studies as a first step of screening GRF analogues. Acylation of the N-terminus of hGRF(1-29)NH_2 generally decreased its affinity (relative affinity to hGRF(1-29)NH_2 (RA), 26-85%). Replacement of the C-terminal carboxamide by a free carboxylic function decreased affinity likely by diminishing its proteolytic stability (RA, 57%). Removal of Tyr^1, Ser^9, Lys^{12}, Val^{13}, Gly^{15}, Gln^{16}, or Lys^{21} drastically decreased its affinity (RA, <3%). Multiple amino acid deletions in the segment 13-21 of hGRF(1-29)NH_2 also led to a loss of affinity as did replacing segment 13-15, 16-18, or 19-21 by an octanoyl moiety (RA, <1%). Removal of Asn^8, Gln^{24}, Asp^{25}, Ile^{26}, Met^{27}, and Ser^{28} or Arg^{29} had less effect on GRF receptor affinity (RA, 5-33%). Removal of Met^{27} or Ser^{28} only slightly affected hGRF(1-29)NH_2 affinity (RA, 62-78%). Altogether, these results indicate that the amino acids contained in the segment 13-21 are more important than those of 24-29 to insure high affinity receptor binding or to maintain an optimal conformation to allow GRF binding.

Introduction

Since the isolation and characterization of growth hormone (GH)-releasing factor (GRF),[2] a number of GRF analogues have been synthesized. Most[3-12] were designed to be potent agonists with potential clinical and zootechnical applications. Their structures were based upon that of hGRF(1-29)NH_2, the N-terminal portion of hGRF(1-44)NH_2, as this portion retains the full potency of the native 44 amino acid peptide to induce GH secretion in vitro and/or in vivo, in various species. Human GRF(1-29)NH_2 also possesses a high degree of sequence homology with porcine, bovine, and ovine GRF(1-29)NH_2 (≥93%) suggesting multiple applications, in various species, for a sole analogue.

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *158*, 9-37). All optically active amino acids are of the L configuration, unless otherwise specified. Additional abbreviations used are as follows: Ac, acetyl; desaminoTyr, 3-(4-hydroxyphenyl)propionic acid.

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Table I. Physicochemical Data

no.[d]	MW	% overall yield	HPLC[a] t_R, min	HPLC[a] % homogeneity (214 nm/280 nm)
1	3358	24	24.0,[b] 21.8[c]	99/100[b,c]
2	3359	29	22.8,[b] 21.0[c]	99/100[b,c]
3	3474	15	19.8,[b] 21.2[c]	97/100[b,c]
4	3400	24	27.2,[b] 24.2[c]	99/100[b,c]
5	3516	10	22.6,[b] 22.4[c]	100/100,[b] 99/97[c]
6	3443	17	25.0,[b] 22.2[c]	98/100[b,c]
7	3471	12	25.4,[b] 22.6[c]	99/100[b,c]
8	3499	15	25.8,[b] 23.6[c]	99/100[b,c]
9	3336	14	20.8,[b] 21.0[c]	99/100,[b] 99/97[c]
10	3209	21	20.9,[b] 21.0[c]	98/100[b,c]
11	3138	23	21.4,[b] 20.8[c]	99/100[b,c]
12	3244	12	25.0,[b] 22.6[c]	100/100[b,c]
13	3271	17	24.8,[b] 22.0[c]	100/100[b,c]
14	3230	8	25.5,[b] 23.6[c]	100/100[b,c]
15	3259	16	20.7,[b] 21.2[c]	99/100[b,c]
16	3301	7	25.9,[b] 26.6[c]	99/100[b,c]
17	3146	13	19.4,[b] 19.0[c]	100/100[b,c]
18	3089	13	18.6,[b] 16.4[c]	100/100[b,c]
19	3230	19	19.2,[b] 18.5[c]	100/100[b,c]
20	3230	17	24.7,[b] 25.8[c]	100/100[b,c]
21	3171	10	21.1,[b] 21.4[c]	98/100[b,c]
22	3074	9	38.3,[b] 36.6[c]	97/100[b,c]
23	3230	8	29.5,[b] 28.6[c]	100/100[b,c]
24	3003	12	29.2,[b] 26.0[c]	100/100[b,c]
25	3144	3	25.0,[b] 21.0[c]	100/100[b,c]
26	2673	6	34.0,[b] 31.2[c]	100/100[b,c]
27	3130	14	22.6,[b] 20.0[c]	100/100[b,c]
28	3243	13	22.8,[b] 20.2[c]	100/100[b,c]
29	3245	13	20.0,[b] 16.7[c]	97/96[b,c]
30	3227	16	21.0,[b] 17.9[c]	100/100[b,c]
31	3271	18	24.2,[b] 22.8[c]	100/100[b,c]
32	3202	13	25.4,[b] 22.6[c]	100/100[b,c]
33	3140	36	19.8,[b] 17.3[c]	100/100[b,c]
34	3357	16	27.4,[b] 24.6[c]	100/100[b,c]
35	3139	21	24.6,[b] 21.1[c]	100/100[b,c]

[a] μBondapak C_{18} (10-μm particles) column (0.39-cm × 15-cm); t_R, retention time. [b] Linear gradient: solvent A consisted of 0.01% aqueous TFA (pH 2.9) and solvent B consisted of CH_3CN/0.01% TFA; 0.67% B/min for 30 min, initial condition 20% B, flow rate 1.5 mL/min, 23 °C. [c] Linear gradient: solvent A consisted of 0.1 M aqueous $NaClO_4$ (pH 2.5) and solvent B consisted of CH_3CN; 0.67% B/min for 45 min, initial condition 30% B, flow rate 1.5 mL/min, 23 °C. [d] Satisfactory amino acid analysis obtained.

However, the potency of these peptides has always been determined in cultured cells of rat adenopituitaries or in living animals. In such assay systems, the molecular features necessary to increase the receptor affinity of GRF

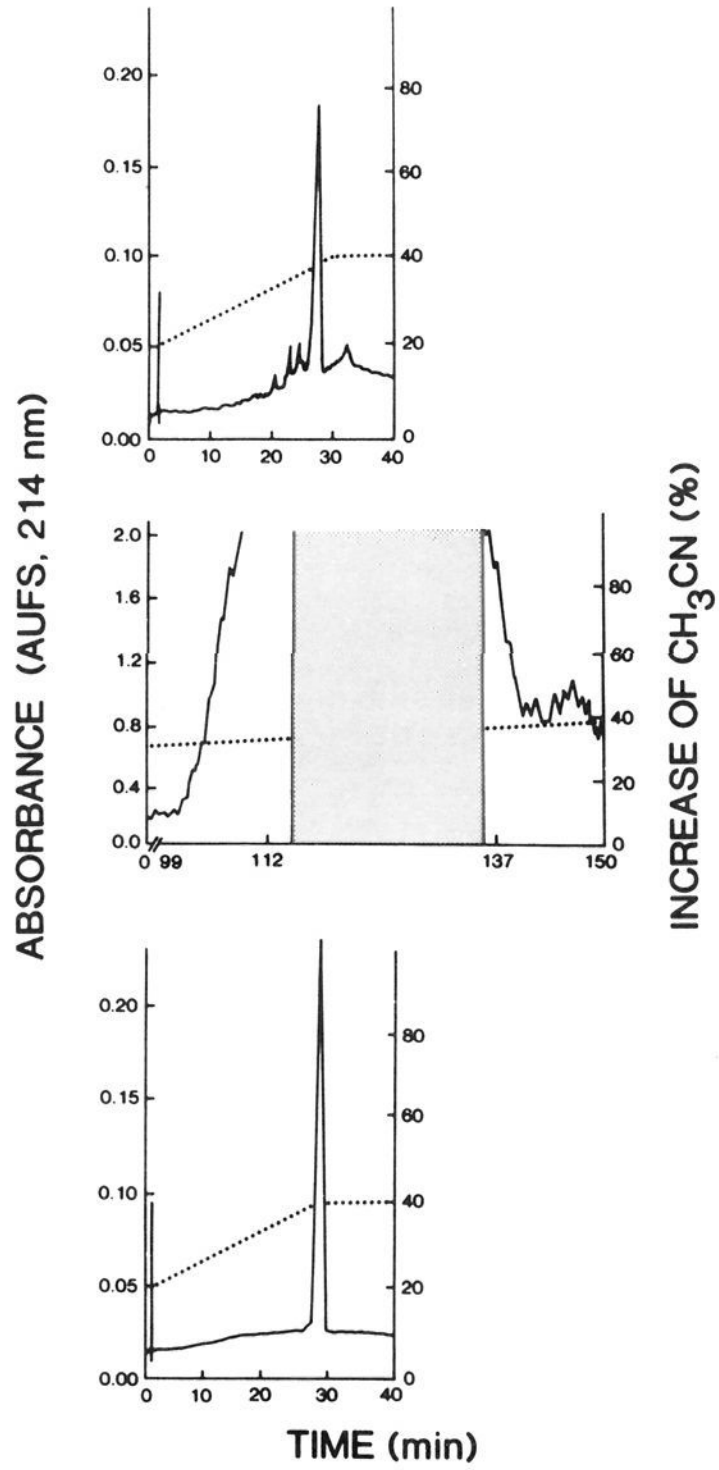

Figure 1. Analytical and preparative chromatographic profiles of [desaminoTyr^1,D-Ala^2,Ala^{15}]hGRF(1–29)NH_2 detected at 214 nm. Top panel, 25 μg of the post HF peptide was subjected to analysis on a C_{18} Bondapak column (0.39-cm × 15-cm) using a binary solvent system. A linear gradient of 0.01% aqueous TFA, pH 2.9 (eluent A) and CH_3CN/0.01% TFA (eluent B) (0.67% B/min for 30 min, initial condition 20% B, flow rate 1.5 mL/min, back-pressure 1650 psi) was used. Middle panel, 4.0 g of the post HF peptide was purified on a Delta Pak column (5.7-cm × 30-cm) using a linear gradient of 0.1% aqueous TFA, pH 1.9 (eluent A) and CH_3CN/0.1% TFA (eluent B) (0.125% B/min for 160 min, initial condition 20% B, flow rate 75 mL/min, back-pressure 650 psi). The dotted area represents homogeneous peptide fractions. Bottom panel, 20 μg of purified [desaminoTyr^1,D-Ala^2,Ala^{15}]-hGRF(1–29)NH_2 was analyzed according to the conditions described in the top panel and showed a purity of 99.5%.

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Table II. Potency of GRF Analogues To Inhibit [^{125}I-Tyr^{10}]hGRF(1–44)NH$_2$ Specific Binding in Rat Adenopituitary and Stability of Peptides in the Binding Assay Conditions[a]

no.	compound	IC$_{50}$,[b] nM	Hill number	RA,[c] %	RC,[d] %
1	hGRF(1–29)NH$_2$	5.6 ± 0.6	0.47 ± 0.02	100	81 ± 4
2	hGRF(1–29)OH	9.8 ± 2.2	0.43 ± 0.03	57	47 ± 3
3	rGRF(1–29)NH$_2$	0.8 ± 0.1	0.64 ± 0.05	700	97 ± 2
4	Ac-hGRF(1–29)NH$_2$	17.6 ± 1.8	0.51 ± 0.01	32	85 ± 3
5	Ac-rGRF(1–29)NH$_2$	1.9 ± 0.5	0.51 ± 0.04	295	94 ± 4
6	[4-aminobutyryl^0]hGRF(1–29)NH$_2$	13.7 ± 3.0	0.52 ± 0.02	41	ND
7	[6-aminohexanoyl^0]hGRF(1–29)NH$_2$	6.6 ± 3.9	0.42 ± 0.04	85	ND
8	[8-aminooctanoyl^0]hGRF(1–29)NH$_2$	21.6 ± 6.2	0.64 ± 0.13	26	100 ± 5
9	rGRF(2–29)NH$_2$	44.2 ± 14.2	0.89 ± 0.01	13	100 ± 4
10	[Ala^{15}]hGRF(2–29)NH$_2$	377 ± 153	0.70 ± 0.07	1.5	ND
11	[Ala^{15}]hGRF(3–29)NH$_2$	311 ± 98	0.44 ± 0.01	1.8	93 ± 6
12	[des Asn^8]hGRF(1–29)NH$_2$	95 ± 28	1.53 ± 0.26	5.9	100 ± 7
13	[des Ser^9]hGRF(1–29)NH$_2$	217 ± 26	1.52 ± 0.18	2.6	ND
14	[des Lys^{12}]hGRF(1–29)NH$_2$	836 ± 26	1.26 ± 0.02	0.7	92 ± 8
15	[des Val^{13}]hGRF(1–29)NH$_2$	>1000			89 ± 2
16	[des Gly^{15}]hGRF(1–29)NH$_2$	1000			88 ± 3
17	[des Val^{13},des Leu^{14}]hGRF(1–29)NH$_2$	>1000			ND
18	[des Val^{13},des Leu^{14}, des Gly^{15}]hGRF(1–29)NH$_2$	>1000			ND
19	[octanoyl$^{13-15}$]hGRF(1–29)NH$_2$	>1000			94 ± 5
20	[des Gln^{16}]hGRF(1–29)NH$_2$	>1000			ND
21	[octanoyl$^{16-18}$]hGRF(1–29)NH$_2$	>1000			ND
22	[des Arg^{20},des Lys^{21}]hGRF(1–29)NH$_2$	>1000			ND
23	[des Lys^{21}]hGRF(1–29)NH$_2$	>1000			ND
24	[des Ala^{19},des Arg^{20}, des Lys^{21}]hGRF(1–29)NH$_2$	1000		0.6	ND
25	[octanoyl$^{19-21}$]hGRF(1–29)NH$_2$	>1000			ND
26	[des Gln^{16},des Leu^{17},des Ser^{18},des Ala^{19},des Arg^{20},des Lys^{21}]hGRF(1–29)NH$_2$	>1000			ND
27	[des Gln^{24}]hGRF(1–29)NH$_2$	45.2 ± 16.6	0.57 ± 0.04	12	85 ± 9
28	[des Asp^{25}]hGRF(1–29)NH$_2$	16.9 ± 3.5	0.53 ± 0.01	33	ND
29	[des Ile^{26}]hGRF(1–29)NH$_2$	20.3 ± 3.4	0.52 ± 0.01	30	ND
30	[des Met^{27}]hGRF(1–29)NH$_2$	9.5 ± 1.1	0.37 ± 0.03	62	ND
31	[des Ser^{28}]hGRF(1–29)NH$_2$	7.2 ± 0.3	0.47 ± 0.03	78	100 ± 6
32	[des Arg^{29}]hGRF(1–29)NH$_2$	20.2 ± 4.2	0.56 ± 0.03	28	ND
33	[des Met^{27},des Ser^{28}]hGRF(1–29)NH$_2$	120 ± 11	0.68 ± 0.03	4.6	94 ± 4
34	[desaminoTyr^1,D-Ala^2,Ala^{15}]hGRF(1–29)NH$_2$	0.73 ± 0.03	0.62 ± 0.04	767	87 ± 6
35	[desaminoTyr^1,D-Ala^2,Ala^{15},des Met^{27},des Ser^{28}]hGRF(1–29)NH$_2$	130 ± 14	0.64 ± 0.04	4.3	93 ± 4

[a] Values represent the mean ± SEM of three experiments performed in duplicate or triplicate. [b] IC$_{50}$: concentration of peptide displacing 50% of specifically bound ^{125}I-GRF as determined by the LIGAND program for analysis of competition studies. [c] RA: relative affinity in percentage compared to hGRF(1–29)NH$_2$. [d] RC: residual concentration of peptides after a 60-min incubation with pituitary homogenates, in the binding assay conditions, as determined by quantitative HPLC.

cannot be fully differentiated from those that improve its proteolytic resistance. To assess the affinity of GRF analogues, we have recently characterized [^{125}I-Tyr^{10}]-hGRF(1–44)NH$_2$ binding to rat pituitary, developing a sensitive and reliable binding assay in which GRF amide-related peptides are stable.[13] In addition to exhibiting a similar affinity to [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)NH$_2$ for the high affinity class of GRF binding sites,[14-16] [^{125}I-Tyr^{10}]hGRF(1–44)NH$_2$ allows the identification of a low affinity class of GRF binding sites.[13,17-19] In the present study, we have evaluated the effect of size reduction, substitutions, or modifications at the termini on the affinity of hGRF(1–29)NH$_2$ for its pituitary plasma membrane binding sites.

Results and Discussion

Peptides 1–35 were synthesized by solid-phase technique using a scheme based on *t*-Boc chemistry/acid labile amino acid protecting groups. BOP was used as the coupling reagent with concomitant neutralization instead of couplings with preformed symmetrical anhydrides.[20-22] This approach constitutes a methodological improvement in structure–function studies of long peptides since it allows

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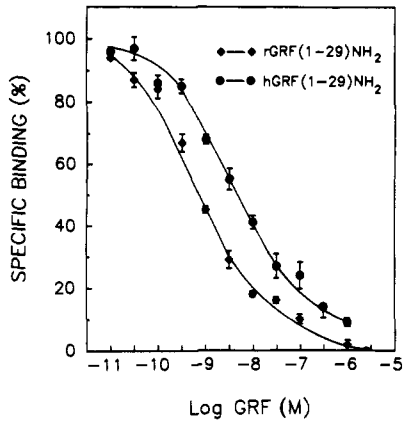

Figure 2. Inhibition of ^{125}I-GRF specific binding to rat adenopituitary homogenate by rGRF(1-29)NH$_2$ and hGRF(1-29)NH$_2$. Values are expressed as percentage of the radioligand specifically bound as a function of GRF molar concentration and represent the mean ± SEM of six independent experiments, each performed in duplicate or triplicate.

less amino acid consumption and shorter cycles without compromising on peptide yield and quality. Purification, accomplished in one step by reverse-phase high-performance liquid chromatography (HPLC) yielded homogeneous products (≥97% as shown in Table I and Figure 1). Amino acid analyses of the pure peptides after acidic hydrolysis confirmed their theoretical composition and assessed their peptide content (data not shown).

The affinity of hGRF(1-29)NH$_2$ (**1**), rGRF(1-29)NH$_2$ (**3**), and that of their analogues is reported in Table II. It was estimated by measuring the concentration of peptide necessary to inhibit 50% of [^{125}I-Tyr^{10}]hGRF(1-44)NH$_2$ specific binding and was 7 times lower for rGRF(1-29)NH$_2$ compared to its human counterpart. Such a difference in the potency of r- and hGRFs in eliciting in vitro GH secretion has been previously documented for rGRF(1-43)OH, rGRF(1-27)NH$_2$, and rGRF(1-23)NH$_2$, which are respectively 3.9, 6.1, and 9.7 times more potent than hGRF(1-44)NH$_2$, hGRF(1-27)NH$_2$, and hGRF(1-23)-NH$_2$.[23] Seifert et al. have reported a similar difference between the in vitro potency of r- and hGRF(1-29)NH$_2$, although when [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1-32)NH$_2$ was used as radioligand, both peptides exhibited a similar binding affinity.[14] On the other hand, the in vitro antagonistic potency of [D-Arg^2]rGRF(1-29)NH$_2$ was only 1.3 times greater than that of its human homologue.[24] The inhibition curves of ^{125}I-GRF by hGRF(1-29)NH$_2$ and rGRF(1-29)NH$_2$ presented in Figure 2 are parallel, indicating that the two compounds bind to the same population of high and low affinity GRF binding sites.[13] This parallelism was conserved for other analogues that inhibited ^{125}I-GRF in a concentration-dependent manner (data not shown). The Hill number, which is lower than 0.8 ± 0.1 for most of the peptides, also suggests that they bind to both GRF binding sites. Whether or not compounds **12-14** bind only to the low affinity sites remains to be documented. Along with the binding affinity, the stability of GRF analogues, in the binding assay conditions, was assessed by quantitative HPLC analysis of their residual concentration (Table II). All the analogues tested, with a C-terminal carboxamide, were relatively stable, since 81-100% of their initial concentration was recovered. This confirms and extends our previous results on the stability of hGRF(1-44) and hGRF(1-29) amidated forms in our binding assay conditions.[13] The sole exception was **2**, the C-terminus free carboxylic form of hGRF(1-29)NH$_2$, whose residual concentration was 47% in these experimental conditions. This is not surprising, since the binding assay medium contained only EDTA, which is not sufficient to inhibit all carboxypeptidase activities that might be present in the pituitary homogenate.

Compounds **2** and **4-8** represent analogues of h- and rGRF(1-29)NH$_2$ that are modified at the C- or N-terminus. Replacement of the C-terminal carboxamide by a carboxylic function (**2**) decreased its affinity 1.7-fold. A similar result was reported regarding the relative potency of hGHF(1-29)OH in inducing GH secretion in vitro.[23,25] Since its effective concentration was also decreased 1.7-fold at the end of the incubation period, it might be suggested that the carboxamide function plays a more important role in proteolytic resistance of GRF than in receptor affinity. However, binding assay conditions in which hGRF(1-29)OH is stable will have to be developed to determine its real affinity. Compounds **4** and **5**, which represent the N$^\alpha$-acetylated form of h- and rGRF(1-29)NH$_2$, had respectively a 3.1 and 2.4 times lower affinity for GRF binding sites than their homologues. A number of studies on the role of N-terminal acetylation of GRF in activating adenylate cyclase or inducing GH secretion in vitro and in vivo, have yielded conflicting results. While it decreased 2-fold the potency of hGRF(1-29)NH$_2$ to activate adenylate cyclase,[5] it respectively increased the in vitro and in vivo hGRF(1-29)NH$_2$-induced GH secretion 2.4- and 12-fold,[8] suggesting that the loss of affinity caused by N$^\alpha$-acetylation of GRFs might be counterbalanced by an increased proteolytic resistance to aminopeptidases in cell culture assays and living animals. N-terminus elongation of hGRF(1-29)NH$_2$ by 4, 6, or 8 carbon atoms (**6-8**) to increase peptide hydrophobicity, while preserving the α-amino function, generated analogues with a similar (**7**) or reduced affinity (**6,8**). Altogether, these results indicate that modifications to increase the hydrophobic character at the N-terminus of hGRF(1-29)NH$_2$ do not constitute a suitable approach to increase receptor affinity.

Peptides **9-35** represent GRFs comprising single or multiple deletions without (**9, 12-18, 20, 22-24, 26-33**) or with substitutions (**10, 11, 19, 21, 25, 34, 35**). Removal of His^1 from rGRF(1-29)NH$_2$ (**9**) or Tyr^1 from [Ala^{15}]-hGRF(1-29)NH$_2$ (**10**) generated peptides that exhibited a similar decreased affinity (60-fold range) compared to their parent peptides, suggesting that the substitution of Gly^{15} by Ala^{15} in the hGRF(1-29)NH$_2$ structure did not have a marked influence on its affinity. It has been reported that such a modification improved the in vitro potency of hGRF(1-29)NH$_2$ to induce GH secretion 5-fold by maximizing the amphiphilic α-helical structure.[12,26] It is not known, however, if such a conformational change could also increase the proteolytic resistance of hGRF(1-

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29)NH_2 to endopeptidases. Removal of the second amino acid at the N-terminus of [Ala^{15}]hGRF(1–29)NH_2 (**11**) did not significantly further influence its affinity. Our results are similar (relative affinity to hGRF(1–44)NH_2, 0.002–0.003) to those of Ling et al.[23] and Campbell et al.[16] showing that the relative in vitro potency and affinity[16] of hGRF(2–44)NH_2, hGRF(3–44)NH_2, and hGRF(3–29)-NH_2 was in the range of 0.001 compared to hGRF(1–44)NH_2. Removal of Asn^8 (**12**) or Ser^9 (**13**) decreased the affinity of GRF 17- and 38-fold, respectively, while deletion of Lys^{12} (**14**) or Gly^{15} (61 ± 1% inhibition of ^{125}I-GRF specific binding at 1 μM) (**16**) shifted its IC_{50} to the micromolar range. Removal of Val^{13} (13 ± 4% inhibition at 1 μM) (**15**), Val^{13} and Leu^{14} (no inhibition at 1 μM) (**17**), or Val^{13}, Leu^{14}, and Gly^{15} (no inhibition a 1 μM) (**18**) reduced the affinity of hGRF(1–29)NH_2 beyond the micromolar range, suggesting that at least Val^{13} is critical for binding or for an optimal conformation that allows binding of other residues to GRF receptors. Restoring the length of **18** to that of hGRF(1–29)NH_2, but not consequently its conformation, with an octanoyl moiety (**19**), generated an inactive peptide (no inhibition at 1 μM), indicating the importance of a specific conformation in this segment of the molecule. This is in agreement with the results of Sato et al.,[7] showing that a D-Val^{13} or a D-Leu^{14} substitution drastically reduces the potency of hGRF(1–29)NH_2 to elicit GH secretion in vitro. Deletion of Gln^{16} (**20**) generated a peptide that inhibited 37 ± 4% of ^{125}I-GRF-specific binding at 1 μM. Replacement of Gln^{16}, Leu^{17}, and Ser^{18} by an octanoyl residue (**21**) or deletion of Arg^{20} and Lys^{21} (**22**) or of the sole Lys^{21} (**23**) generated analogues devoid of affinity for GRF binding sites at up to 1 μM. Deletion of the segment 19–21 of hGRF(1–29)NH_2 (**24**) resulted in a compound with an IC_{50} of 1 μM. Its replacement by an octanoyl residue (**25**) did not restore the affinity of this peptide to that of hGRF(1–29)NH_2. Deletion of the segment 16–21 (**26**) resulted in a compound with no affinity for the GRF binding sites. Deletion of Gln^{24} (**27**), Asp^{25} (**28**), Ile^{26} (**29**), Met^{27} (**30**), Ser^{28} (**31**), or Arg^{29} (**32**) resulted in compounds that retained nanomolar affinity, relative affinity of **30** and **31** to that of hGRF(1–29)NH_2 being 62% and 78%, respectively. Our present results contrast in some ways with those of Ling et al.[23] The order of potency of single amino acid deleted analogues of hGRF(1–27)NH_2 is des 25 > des 26 > des 16 > des 15 > des 8 > des 9 > des 24 > des 12 > des 1 > des 2 > des 13 > des 21[16] while the receptor affinity is des 25 > des 26 > des 24 > des 8 > des 9 > des 2 ≥ des 1 > des 12 > des 15 > des 13 ≈ des 16 ≈ des 21. This discrepancy may arise from structural differences between the two molecules and/or from the type of assay used. [DesaminoTyr^1,D-Ala^2,Ala^{15}]hGRF(1–29)NH_2 (**34**), a peptide initially designed to maximize the amphiphilic secondary structure of hGRF(1–29)NH_2 in position 15 and to improve its proteolytic resistance,[25] had a 7.7 times greater affinity than hGRF(1–29)NH_2, indicating that substitutions in position 1 and 2 also increased binding affinity. Deletion of Met^{27} and Ser^{28} (**33**) induced a substantial decrease of affinity, suggesting that multiple deletions of individual amino acids that do not have a prominent role in the affinity of hGRF(1–29)NH_2 may influence its optimal conformation for binding to GRF receptors. Finally, the introduction of selective substitutions in peptide **33** (**35**) that increased GRF(1–29)NH_2 affinity, such as in **34**, was not effective in a 27 amino acid structure.

Receptor affinity, biological potency, and metabolic stability represent three parameters that must be optimized in order to develop potent agonists. It is therefore of prime importance to have simple assay systems to evaluate them independently. The [^{125}I-Tyr^{10}]hGRF(1–44)NH_2 binding assay constitutes an effective method of determining the affinity of hGRF(1–29)NH_2 analogues for their specific pituitary binding sites. It allowed us to find that [des Met^{27}]- and [des Ser^{28}]hGRF(1–29)NH_2 exhibit a similar affinity to that of hGRF(1–29)NH_2. These results suggest that size reduction alone, or with concomitant amino acid modifications that restore or preserve high-affinity binding, could lead to a new generation of GRF analogues.

Experimental Section

Materials. *p*-Methylbenzhydrylamine resin (0.55 mequiv of amine/g), *p*-(chloromethyl)poly(styrene-co-divinylbenzene 1%) resin (0.65 mequiv of chlorine/g) and N^{α}-*tert*-butyloxycarbonyl (Boc) protected amino acid derivatives were purchased from Bio-Mega Inc. Amino acid side chains were protected as follows: Asp, β-*O*-Bzl; Thr, *O*-Bzl; Ser, *O*-Bzl; Tyr, *O*-2-Br-Z; Lys, *N*-2Cl-Z; Arg, N^{g}-Tos. Prior to use, all amino acid derivatives were tested for purity by thin-layer chromatography and melting point determination. N^{α}-Boc-8-aminooctanoic acid, N^{α}-Boc-6-aminohexanoic acid, and N^{α}-Boc-4-aminobutyric acid were N^{α}-acylated by means of di-*tert*-butyl dicarbonate.[27,28] Reagent-grade 2-propanol and methylene chloride (CH_2Cl_2) were bought from Anachemia Canada Inc. CH_2Cl_2 was distilled from anhydrous sodium carbonate. *N,N*-Diisopropylethylamine (DIEA) and 4-methylmorpholine (Aldrich Chemicals) were distilled from ninhydrin and subsequently kept at 4 °C. *N,N*-Dimethylformamide (DMF) (Anachemia Canada Inc.) was distilled from ninhydrin, in vacuo, after a 3-day storage over 4-A molecular sieves and kept under an argon atmosphere. Anisole (Aldrich Chemicals) was distilled and kept at 4 °C. Trifluoroacetic acid (TFA) (Halocarbon Products Co.) and acetic acid (HOAc) (Anachemia Canada Inc.) were distilled prior to use. *N,N'*-Dicyclohexylcarbodiimide (DCC), acetic anhydride (Ac_2O), and 1-hydroxybenzotriazole (HOBT) (Aldrich Chemicals), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (Richelieu Biotechnologies) and accusolve-grade methanol (MeOH) and acetonitrile (CH_3CN) (Anachemia Canada Inc.) were used without further purification.

Peptide Synthesis. Benzhydrylamine resin was coupled to Boc-Arg (N^{g}-Tos) or Boc-Ser (*O*-Bzl) after neutralization in 5% DIEA. Merrifield resin[29] was coupled to Boc-Arg (N^{g}-Tos), by using the cesium salt method.[30] The degree of substitution was determined by the picric acid test[31] and was 0.41 mmol of amino acyl/g. Compounds **1–35** were prepared by automated solid-phase synthesis (Vega 250). Couplings of Boc amino acid derivatives (3 equiv) except for Boc-Asn and Boc-Gln were achieved with BOP (3 equiv),[20–22] using in situ neutralization (6 equiv, DIEA); the latter two (3 equiv) were coupled with DCC/HOBT (3 equiv); 4-methyl morphiline (0.5 equiv) was added 30 min after beginning of the coupling when a second coupling was needed. Their completion was ascertained by a ninhydrin colorimetric test.[32] Boc protecting groups were removed with TFA–CH_2Cl_2 (40:60) containing 1% D,L-methionine (w/v) when Boc-Met was incorporated

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in the growing peptide. This was followed by neutralization with DIEA/CH_2Cl_2 (5:95) when the DCC/HOBT method was used. After completion of the synthesis and removal of the last Boc group, peptides 4 and 5 were N-acetylated with Ac_2O. Deprotection of the amino acid side chains and cleavage of the peptides from the resin were performed with anhydrous hydrogen fluoride (HF), anisole (9:1, v/v; 10 mL/g of peptide–resin intermediate) at −15 °C for 30 min and then at 0 °C for 30 additional minutes. When Met was present in the peptide chain, 0.5% D,L-methionine (w/v) was added to the reaction mixture. HF removal was done in vacuo followed by precipitation of the crude peptides with peroxide-free anhydrous ethyl ether and solubilization with 20% aqueous N_2-purged HOAc. Solutions were lyophilized to yield amorphous powders.

Peptide Purification and Physicochemical Characterization. The crude peptides were submitted to analytical HPLC to optimize the purification procedure. Then, a sample load ranging from 100 mg to 4 g was subjected to preparative HPLC on a Partisil 10 ODS-3 Whatman (10-μm particle size) column (2.2-cm × 50-cm) or on a Delta-Pak C_{18} Waters (15-μm particle size) column (5.7-cm × 30-cm), using a binary solvent system consisting of 0.01% aqueous TFA (eluent A), pH 2.9, and CH_3CN/0.01% TFA (eluent B) and appropriate gradients. The concentration of TFA was increased to 0.1% in both mobile phases, when gram quantities were purified. A flow rate of 7.0–8.0 mL/min was used with the Whatman column, while 75 mL/min was used with the Delta-Pak column. A typical chromatographic run for [desaminoTyr[1],D-Ala[2],Ala[15]]hGRF(1–29)NH_2, using the Whatman column, was as follows: 0.2% B/min for 100 min, initial condition 20% B, flow rat 8.0 mL/min. A chromatographic profile of the same peptide, using a Delta-Pak column, is shown in Figure 1 (middle panel). Elution of the peptides was monitored at 214 or 280 nm. Collected fractions were readily screened by analytical HPLC and pooled accordingly. The peptides thus obtained were subjected to rotary evaporation, in vacuo, to remove CH_3CN and then lyophilized twice. Purified peptides were analyzed for homogeneity by analytical HPLC on a μBondapak C_{18} (10-μm particles) column (0.39-cm × 15-cm) using appropriate linear gradients of 0.01% aqueous TFA, pH 2.9, and 0.01% TFA/CH_3CN and of 0.01 M $NaClO_4$, pH 2.5, and CH_3CN (Table I and Figure 1). Their amino acid composition and peptide content were assessed by quantitative amino acid analysis after acidic hydrolysis in vacuo (6 N HCl, 110 °C, 18 h) as previously described.[33] Individual amino acid recovery ranged from 0.82 to 1.12 per residue.

GRF Binding Assay. Pituitary homogenate and competition studies were performed as previously described,[12] using [[125]I-Tyr[10]]hGRF(1–44)NH_2 (Amersham; 2000 Ci/mmol) as radioligand and 2-month-old male Sprague-Dawley rat (Charles River, Canada). Briefly, freshly dissected anterior pituitaries were rinsed and then homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 5 mM EDTA (1 pituitary per 0.5 mL). Competition studies were performed using 50 μL of homogenate (70–75 μg of protein, as determined by the Lowry method[34]), 35–50 pM [125]I-GRF, and increasing concentrations of GRFs (0–1000 nM) or with 2.4 μM rGRF(1–29)NH_2 for determination of nonspecific binding, in a total volume of 300 μL of Tris-HCl buffer, pH 7.4, containing 5 mM EDTA, 5 mM $MgCl_2$, and 0.42% BSA. Incubations were carried out at 23 °C for 60 min and stopped by centrifugation (12000*g*, 5 min, at 4 °C). Supernatants were then aspirated and tube tips were cut off and counted for radioactivity in a gamma counter. The LIGAND computerized program was used to analyse all competition curves.[35]

Degradation Assay of GRFs in Pituitary Homogenates. Resistance of hGRF(1–29)NH_2, rGRF(1–29)NH_2, and their analogues to proteolysis was investigated by HPLC in the binding assay conditions as previously described.[12] Briefly, pituitary homogenates (150 μg of protein/100 μL) were incubated with 10 μM of each peptide in a final volume of 500 μL at 23 °C for 0 and 60 min. The degradation reaction was stopped by the addition of 300 μL cold 50 mM phosphate solution, pH 0.8, and immediate centrifugation (12000*g*, 5 min, 4 °C). Parallel experiments were conducted without pituitary homogenates to control for GRF adsorption. The residual concentrations of peptides were analyzed by HPLC on a μ-Bondapak C_{18} (10-μm particles) column (0.39-cm × 15-cm) using a Waters instrument equipped with an automated injector, a gradient controller, and a UV detector (214 nm) coupled to a Spectra-Physics SP 4270 integrator. The mobile phase was composed of 0.01% aqueous TFA, pH 2.9 (eluent A), and CH_3CN–0.01% TFA (eluent B). A linear gradient of 0.67% increase of eluent B per min, for 30 min, was used with 20% of B as initial condition and a flow rate of 1.5 mL/min. Quantification was achieved by surface integration. Protein dilution followed by immediate centrifugation efficiently stopped the peptide degradation. This was shown by equivalent peptide recoveries for time zero of incubation with or without membranes.

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