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ISOLATION AND CHARACTERIZATION OF THE BOVINE HYPOTHALAMIC GROWTH HORMONE RELEASING FACTOR

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SUMMARY. A 44 amino acid peptide with high intrinsic growth hormone releasing activity was isolated from 500 bovine hypothalami by means of acid extraction, immunoaffinity chromatography, gel filtration, and two steps of reverse phase HPLC. The growth hormone releasing factor was structurally characterized by gas phase sequence analysis. Reverse phase liquid chromatography of the native peptide and synthetic replicates showed that the molecule possesses an amide rather than a free acid at its carboxyl terminus. The structure of the peptide was established as: 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-Asn-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Lys-Val-Arg-Leu-NH_2 using approximately 2 nmol of material.

The hypothalamic regulation of pituitary growth hormone release is mediated by both positive (growth hormone releasing factor, GRF) and negative (somatostatin) peptide effectors. While the amino acid sequence of the tetradecapeptide, somatostatin, was elucidated in 1973 (1) the minute quantities of GRF in the hypothalamus prevented its structural characterization until quite recently. To date five structurally similar peptides with high intrinsic growth hormone releasing activity have been reported in the literature (2-9). Three such human pancreas tumor-derived peptides possessing 44 (hpGRF-44), 40 (hpGRF-40) and 37 (hpGRF-37) amino acids in identical sequences from their amino termini were the first to be described (2-4). hpGRF-44 is the only structure possessing an amidated carboxyl terminus (hpGRF-40 and hpGRF-37 have

ABBREVIATIONS:
hpGRF-44, hpGRF-40 and hpGRF-37 = human pancreatic growth hormone releasing factors composed of 44, 40 and 37 amino acids, respectively; pGRF-44 = porcine hypothalamic growth hormone releasing factor; rGRF-43 = rat hypothalamic growth hormone releasing factor; bGRF-44 = bovine hypothalamic growth hormone releasing factor; irGRF = immunoreactive growth hormone releasing factor; TEAP = triethylammonium phosphate; HFBA = heptafluorobutyric acid; TFA = trifluoroacetic acid.

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free carboxyl termini) thus suggesting that the smaller fragments result from proteolytic degradation of hpGRF-44. Porcine hypothalamic GRF (pGRF-44) (9) has been shown to be a 44 amino acid, carboxyl-terminally amidated peptide while the rat hypothalamic GRF (rGRF-43) (8) contains only 43 amino acids and possesses a free carboxyl terminus which may be indicative of an incomplete structure. We have recently developed a very simple and highly efficient isolation scheme employing immunoaffinity chromatography which permits the rapid isolation of hypothalamic GRF from many different sources. This methodology has been used in the isolation of both porcine (9) and bovine (bGRF-44) hypothalamic GRFs. This report describes the purification of bGRF-44 and its structural elucidation by gas phase sequence analysis.

METHODS

Immunoaffinity chromatography. Approximately 500 lyophilized bovine hypothalamic median eminence fragments (9.3g) were boiled in water (8 ml/g tissue) for three minutes to inactivate endogenous proteases, cooled to 0°C, homogenized with an equal volume of 0.6 M HCl, 0.5% 2-mercaptoethanol containing phenyl methylsulfonyl fluoride and pepstatin A (10 μg/ml each) and centrifuged. After re-extraction of the pellet the combined supernatants were defatted with petroleum ether: ether (2:1), the ionic strength lowered by the addition of an equal volume of 40 mM sodium phosphate and the pH adjusted to 7.4 with sodium hydroxide. This liquid was pumped through an immunoaffinity column (3.5 x 7.2 cm, V_{bed} = 70 ml) prepared by coupling purified IgG (10) raised against hpGRF-40 (11) to Affi-gel 10 (BioRad Laboratories) according to the manufacturer's recommendations. After washing off the unbound material with 20 mM sodium phosphate/145 mM sodium chloride/0.01% sodium azide, pH 7.4, the adsorbed immunoreactive GRF (irGRF) was eluted with 1 M acetic acid.

Radioimmunoassay, bioassay and structural characterization. During the peptide purification, column effluents were monitored for the presence of GRF by radioimmunoassay using an antiserum (456-12) which was part of a pool of antisera used for immunoaffinity chromatography. Isolated bGRF-44 and its synthetic replicate were assayed for their ability to stimulate growth hormone release from rat pituitary cells in monolayer culture as described (12) and compared for potency with hpGRF-44. Amino acid analyses (13) and the sequence determination (14) were performed as previously described.

RESULTS AND DISCUSSION

A five step purification procedure involving acid extraction, immunoaffinity chromatography, gel filtration and semipreparative and analytical reverse phase HPLC was used to isolate bGRF-44. The high cross-reactivity between anti-hpGRF-40 antibodies and bGRF-44 made immunoaffinity chromatography a very efficient step for the purification of bGRF-44 from crude extracts. Eleven μg of immunoreactive GRF (irGRF) from 500 bovine hypothalami

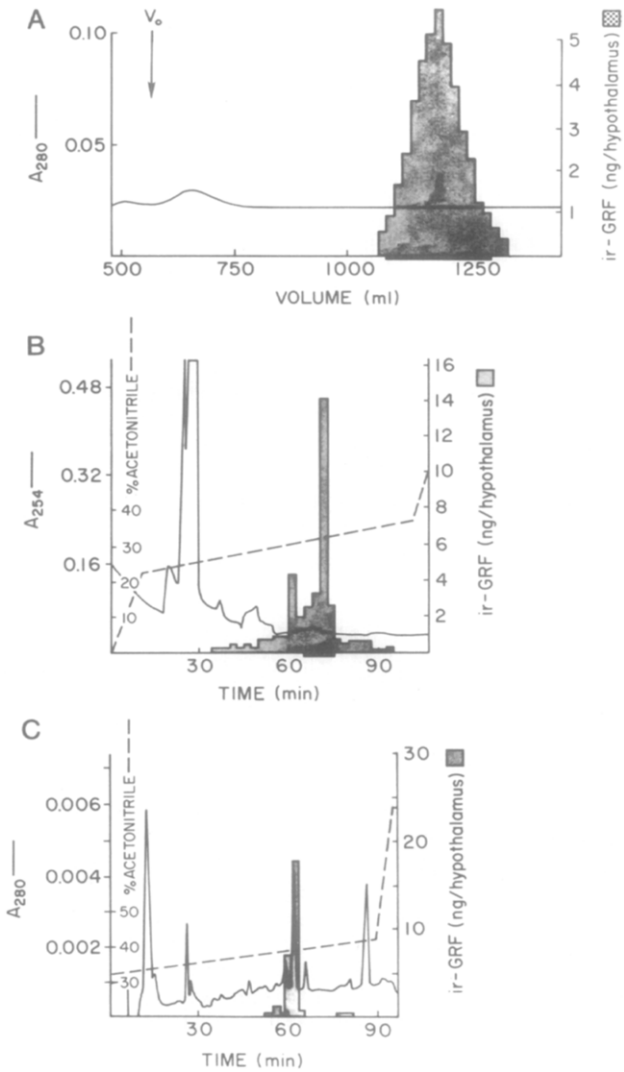

FIGURE 1: Isolation of bGRF-44.

A) The immunoaffinity-purified irGRF was chromatographed over a Sephadex G-75 column (4.5 x 117 cm) equilibrated and developed in 1 M acetic acid/0.2% 2-mercaptoethanol (v/v) at 1 ml/min.

B) The gel-filtered irGRF-containing fractions were pooled (ca. 350 ml) and pumped onto a semi-preparative C18 column (Altex Ultrasphere, 5 μm particle size, 1 X 25 cm) utilizing a 0.25 M triethylammonium phosphate (TEAP), pH 3.0/acetonitrile mobile phase. Fractions of 2.5 ml were collected at 1 ml/min.

C) The major irGRF species from step B was purified to homogeneity by chromatography on an analytical C18 column (Brownlee RP300, 7 μm particle size, 0.46 x 25 cm) using a 0.2% (v/v) heptafluorobutyric acid (HFBA)/acetonitrile solvent system. Fractions of 2.5 ml were collected at 1 ml/min.

At all chromatography steps aliquots of column fractions were subjected to radioimmunoassay after drying in a vacuum centrifuge (Savant) in the presence of 100 μg serum albumin. Horizontal bars denote fractions selected for further purification.

were quantitatively retained by the column. The results of subsequent purification steps are illustrated in Figure 1. Gel filtration (Figure 1A) yielded a single irGRF zone, which upon semipreparative HPLC (Figure 1B), was separated into two irGRF fractions. Rechromatography of the major fraction on an analytical reverse phase HPLC system with different solute selectivity (Figure 1C) yielded a single irGRF peak corresponding to a distinct UV-light-absorbing peak of peptide material. This was subjected to structural characterization and represented 2.1 nmol of bGRF-44. The structure of the minor irGRF (Figure 1B) is unknown; it may represent a degradation product similar to those found in a human pancreas tumor (2-4) or it may be an oxidized form of bGRF-44.

The amino acid composition of the major irGRF (Table I) suggested a large degree of structural homology with both hpGRF-44 and pGRF-44. Direct Edman degradation of 1780 pmol of intact bGRF-44 by gas phase sequence analysis yielded the primary structure of the amino terminal 43 amino acids of the peptide (Table II). The identification of the carboxyl terminal leucine was de-

TABLE I

AMINO ACID COMPOSITION OF bGRF-44[a]

Amino Acid	bGRF-44 (n=2)	
Asx	5.19 ± 0.09	(5)[b]
Thr	0.99 ± 0.02	(1)
Ser	1.57 ± 0.12	(2)
Glx	7.96 ± 0.01	(8)
Gly	2.98 ± 0.11	(3)
Ala	3.89 ± 0.08	(4)
Val	2.34 ± 0.04	(2)
Met	0.73 ± 0.06	(1)
Ile	1.82 ± 0.02	(2)
Leu	5.13 ± 0.04	(5)
Tyr	1.94 ± 0.01	(2)
Phe	0.93 ± 0.06	(1)
His	0	
Trp	0	
Lys	3.24 ± 0.06	(3)
Arg	5.26 ± 0.09	(5)
Cya[c]	0	
Pro	0	

a Values are means (± standard deviation) from n determinations and are not corrected for hydrolysis losses.

b Values in parentheses correspond to actual integer values of the amino acids in bGRF-44.

c Cysteine was determined as cysteic acid.

TABLE II

Gas Phase Sequence Analysis of bGRF-44

Cycle No. (N)	Residue No.	>PhNCS-AA	Yield (pmol)	Carryover (pmol)
1	1	Tyr	628	-
2	2	Ala	588	22.6
3	3	Asp	501	63.7
4	4	Ala	657	45.8
5	5	Ile	483	35.4
6	6	Phe	538	30.6
7	7	Thr	219	33.7
8	8	Asn	304	38.9
9	9	Ser	67.9	23.8
10	10	Tyr	347	20.9
11	11	Arg	417	53.6
12	12	Lys	280	133
13	13	Val	248	47.1
14	14	Leu	273	55.8
15	15	Gly	98.4	40.9
16	16	Gln	120	32.8
17	17	Leu	185	41.1
18	18	Ser	18.9	73.9
19	19	Ala	70.7	2.25
20	20	Arg	128	39.5
21	21	Lys	51.5	84.6
22	22	Leu	108	21.8
23	23	Leu	149	-
24	24	Gln	67.0	84.9
25	25	Asp	64.3	28.2
26	26	Ile	86.6	26.2
27	27	Met	56.2	40.6
28	28	Asn	43.2	37.1
29	29	Arg	87.4	17.6
30	30	Gln	41.5	37.2
31	31	Gln	61.5	-
32	32	Gly	29.0	30.5
33	33	Glu	31.9	13.9
34	34	Arg	66.6	16.7
35	35	Asn	29.6	38.0
36	36	Gln	30.5	13.0
37	37	Glu	28.0	21.6
38	38	Gln	38.9	21.2
39	39	Gly	34.0	26.6
40	40	Ala	19.8	30.9
41	41	Lys	7.65	9.49
42	42	Val	6.10	4.27
43	43	Arg	13.4	2.18
44[a]	44	X	-	10.1

Amount Applied: 1780 pmol Average Repetitive Yield: 91.4%
Initial Yield: 13.8%

[a] The carboxyl terminal leucine was identified by comparison of the above sequence for bGRF-44(1-43) with the amino acid composition for bGRF-44.

duced by comparing the established sequence of bGRF-44(1-43) with the amino acid composition of bGRF-44. The nature of the carboxyl terminus was established by high pressure liquid chromatography studies in which the native peptide was co-chromatographed with synthetic replicates possessing either an

amidated or a free carboxyl terminus. These studies were performed with two different solvent systems and clearly show co-elution of native bGRF-44 with synthetic bGRF-44-NH_2 and separation from synthetic bGRF-44-OH (Figure 2). The entire structural characterization of bGRF-44 required less than 2.1 nmol peptide. Preliminary evaluation of the in vitro bioactivity of both the isolated bGRF-44 and its synthetic replicate in the rat pituitary cell culture system indicates that the molecule has the full intrinsic activity of hpGRF-44.

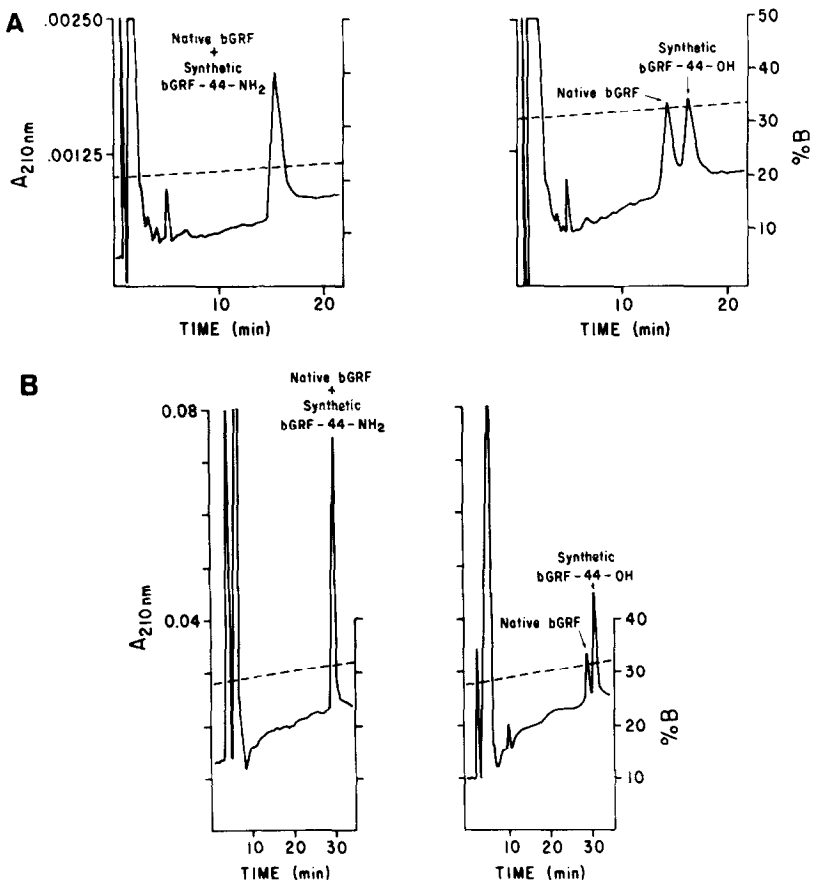

FIGURE 2: Reverse phase liquid chromatography of native bGRF-44 and synthetic replicates containing either a free acid or an amidated carboxyl terminus. A) Solvent A, 0.25 M TEAP, pH 3.0; Solvent B, 20% 0.25 M TEAP, pH 3.0 and 80% acetonitrile. Elution at 1.5 ml/min was performed with a 40 min linear gradient from 31% to 35% B on a Beckman RPSC column (5 μm particle size, 0.46 x 7.5cm). B) Solvent A, 0.1% TFA; Solvent B, 0.1% TFA and 99.9% acetonitrile. Elution at 1.0 ml/min was accomplished with a 60 min linear gradient from 28% to 35% B on a Brownlee Aquapore 300 column (10 μm particle size, 0.46 x 25 cm). All chromatography runs were performed at room temperature with 50 pmol quantities of each peptide.

FIGURE 3: Primary structures of hpGRF-44, pGRF-44, bGRF-44 and rGRF-43. Sequence differences from the human structure are outlined.

The structures of peptides with high intrinsic growth hormone releasing activity have now been established from four different species as shown in Figure 3. The human, porcine and bovine sequences each possess a carboxyl terminally amidated 44 amino acid structure. Surprisingly, rat hypothalamic GRF contains only 43 amino acids and is not amidated. It is possible that rGRF-43 may have been a product of limited in vivo or in vitro degradation and an amidated rGRF-44 may also exist in rat hypothalamus. We have, in fact, found multiple forms of GRF, some of which are carboxyl terminally degraded, in most GRF-containing tissues thus far studied including rat hypothalamus (unpublished observation). Hence, the primary sequences of hpGRF, pGRF and bGRF suggest that the consensus structure of the typical, intact, mature GRF is that of a C-terminally amidated, 44 amino acid peptide hormone.

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REFERENCES

1. Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J. and Guillemin, R. (1973) Science 179, 77-79.
2. Guillemin, R., Brazeau, P., Böhlen, P., Esch, F., Ling, N. and Wehrenberg, W.B. (1982) Science 218, 585-587.
3. Böhlen, P., Brazeau, P., Esch, F., Ling, N., Wehrenberg, W. and Guillemin, R. (1983) Reg. Peptides 6, 343-353.
4. Esch, F., Böhlen, P., Ling, N., Brazeau, P., Wehrenberg, W. and Guillemin, R. J. Biol. Chem. 258, 1806-1812.
5. Esch, F., Böhlen, P., Ling, N., Brazeau, P., Wehrenberg, W., Thorner, M., Cronin, M. and Guillemin, R. (1982) Biochem. Biophys. Res. Commun. 109, 152-158.
6. Rivier, J., Spiess, J., Thorner, M. and Vale, W. (1982) Nature 300, 276-278.
7. Spiess, J., Rivier, J., Thorner, M. and Vale, W. (1982) Biochem. 21, 6037-6040.

8. Spiess, J., Rivier, J. and Vale, W. (1983) Nature 303, 532-535.
9. Böhlen, P., Esch, F., Brazeau, P., Ling, N. and Guillemin, R. (1983) Biochem. Biophys. Res. Commun. 116, 726-734.
10. Garvey, J., Cremer, N. and Sussdorf, D. (1977) Methods in Immunology, 3rd ed. p.218, Addison-Wesley Inc., Benjamin, New York.
11. Böhlen, P., Brazeau, P., Bloch, B., Ling, N., Gaillard, R. and Guillemin, R. (1983) Biochem. Biophys. Res. Commun. 114, 930-936.
12. Brazeau, P., Ling, N., Böhlen, P., Esch, F., Ying, S., and Guillemin, R. (1982) Proc. Natl. Acad. Sci. USA 79, 7909-7913.
13. Böhlen, P. and Schroeder R. (1982) Anal. Biochem. 126, 144-156.
14. Esch, F. (1983) Anal. Biochem. 133 (in press).