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ISOLATION AND CHARACTERIZATION OF THE PORCINE 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 2500 porcine hypothalami by means of acid extraction, immunoaffinity chromatography, gel filtration, and 2 steps of reverse phase HPLC. The growth hormone releasing factor was structurally characterized by gas phase sequence analyses of the intact peptide and its carboxyl terminal cyanogen bromide digestion fragment. 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-Ser-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Arg-Val-Arg-Leu-NH_2 using approximately 6 nmol of material.

Peptides with high intrinsic growth hormone releasing activity have been isolated and structurally characterized from two separate human pancreatic tumors (1-6) as well as from rat hypothalami (7). Three such human pancreas 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 (1,3). hpGRF-44 is the most potent (1,8) of these in an *in vitro* bioassay and is the only structure possessing an amidated carboxyl terminus (hpGRF-40 and hpGRF-37 have free carboxyl termini) thus suggesting that the smaller fragments probably result from proteolytic degradation of hpGRF-44. The primary structure of the 43 amino acid rat hypothalamic peptide (rGRF-43) shows 14 amino acid sequence differences from that of hpGRF-44 and possesses a free carboxyl terminus which may be indicative of an incomplete structure. This possibility is given further support by our characterization, reported herein,

ABBREVIATIONS:

hpGRF-44, hpGRF-40 and hpGRF-37 = human pancreas 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; TEAP = triethylammonium phosphate; HFBA = heptafluorobutyric acid; TFA = trifluoroacetic acid.

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of the 44 amino acid porcine hypothalamic growth hormone releasing factor (pGRF-44) which contains an amidated carboxyl terminus.

MATERIALS and METHODS

Cyanogen bromide was obtained from Eastman. Pyridine, formic acid and n-propanol were each distilled over ninhydrin (1g/l) and under nitrogen. Acetic acid was distilled under nitrogen. Solvents for the Applied Biosystems Model 470A sequencer, ethyl acetate, n-butyl chloride (Burdick & Jackson) and methanol (Matheson, Coleman & Bell) were purified and prepared as described (9) as were three of the four reagents, trifluoroacetic acid (Fluka & Pierce), 1N HCl in methanol and 25% trimethylamine/water. The 15% phenylisothiocyanate/heptane reagent was obtained from Applied Biosystems.

Peptide isolation. Approximately 2500 porcine hypothalamic fragments (lyophilized and stored under vacuum for 12 years; 87 g) were processed in batches of 500 or 1000 hypothalami. Tissue was 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 containing phenylmethylsulfonyl fluoride and pepstatin A (10 µg/ml each) and centrifuged. After re-extraction of the pellet the supernatants were combined, defatted with petroleum ether : ether (2:1), the ionic strength lowered by addition of an equal volume of 40 mM sodium phosphate and the pH adjusted to 7.6 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 pGRF was eluted with 1M acetic acid. Final purification of pGRF was achieved by gel filtration on Sephadex G-75 and with reverse-phase HPLC using the following chromatographic systems: (a) a semipreparative C18 column (Altex Ultrasphere, 5 µm particle size, 1 x 25 cm) with 0.25 M triethylammonium phosphate (TEAP), pH 3.0/acetonitrile as the mobile phase; (b) an analytical C18 column (Brownlee RP-300, 10 µm particle size, 0.46 x 25 cm) with 0.2% (v/v) heptafluorobutyric acid (HFBA)/acetonitrile as the solvent system. Further details are given in the figure legends.

Structural characterization. Amino acid analyses (12) and sequence determinations (3) were performed as previously described. Cyanogen bromide digestion of isolated pGRF and HPLC purification of the carboxyl terminal digestion fragment was carried out as detailed elsewhere (3). To ascertain whether the carboxyl terminal residue was present as the amidated or the free acid form, two synthetic replicates of pGRF-44, one with a free acid at the carboxyl terminus and the other with an amidated carboxyl terminus were made using solid-phase methodology (1). Natural pGRF was compared to both synthetic forms of pGRF on two highly resolutive reverse-phase HPLC systems: (a) an Altex Ultrapore RPSC column (5 µm particle size, 0.46 x 7.5 cm) with TEAP/acetonitrile as mobile phase and (b) a Brownlee Aquapore RP-300 column using 0.1% (v/v) trifluoroacetic acid (TFA)/acetonitrile for elution (13).

Radioimmunoassay and bioassay. Column effluents were monitored for the presence of GRF by radioimmunoassay using an antiserum (456-12) which was part of a pool of antisera for immunoaffinity chromatography (manuscript in preparation). Isolated pGRF and its synthetic replicate were assayed for their ability to stimulate growth hormone release from rat pituitary cells in monolayer culture as described (8) and compared for potency with hpGRF-44.

RESULTS and DISCUSSION

A 5-step purification procedure, involving acid extraction, immunoaffinity chromatography, gel filtration, and semipreparative and analytical reverse

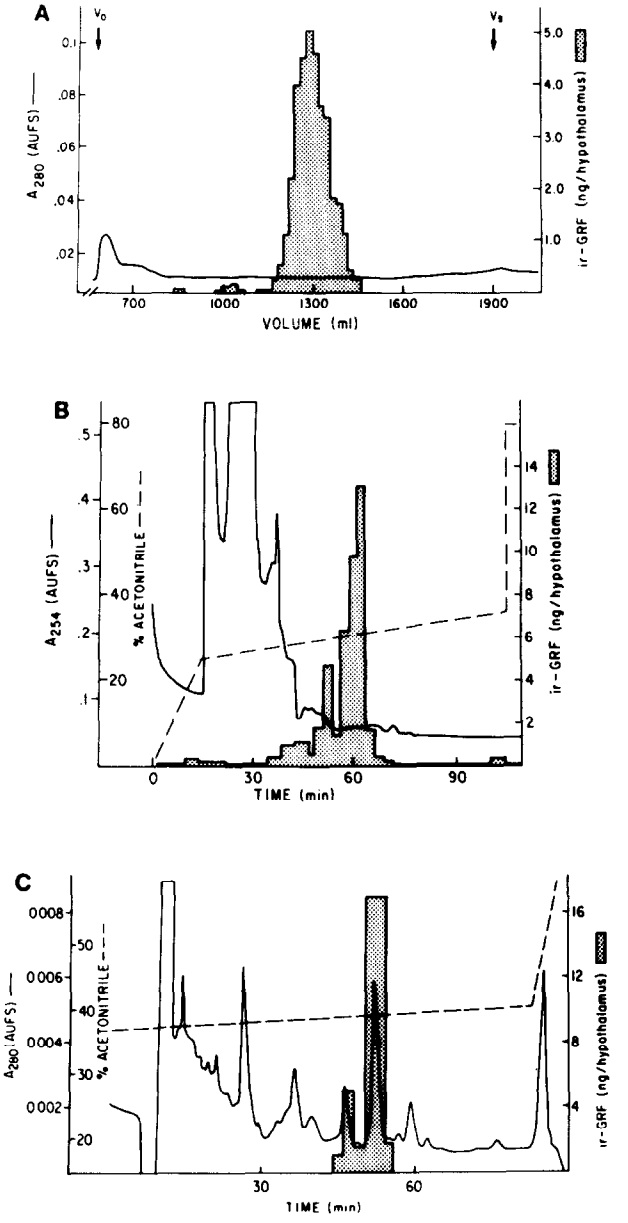

Figure 1. Isolation of pGRF-44 from a batch of 1000 hypothalami. A. Sephadex G-75 gel filtration chromatography of the immunoaffinity-purified porcine hypothalamic acid extract. Column dimensions: 117 x 4.5 cm; the sample (60 ml) was eluted at room temperature with 1 M acetic acid/0.2% (v/v) β-mercaptoethanol at a flow rate of 60 ml/h; fractions of 15 ml were collected and aliquots were subjected to radioimmunoassay after drying in a Speed-Vac centrifuge in the presence of 100 µg serum albumin. B: Semi-preparative reverse phase HPLC of pooled, immunoreactive fractions from gel filtration. The sample (ca. 350 ml) was loaded by pumping it onto the column prior to starting the elution gradient. Fractions of 2.5 ml were collected at a flow rate of 1 ml/min. Aliquots for radioimmunoassay were prepared as described in Fig. 1A. C. Analytical reverse phase HPLC of the major immunoreactive pGRF-44 from a semi-preparative reverse phase HPLC. Other conditions were as in Fig. 1B.

phase HPLC was used to achieve the isolation of pGRF-44. The high cross-reactivity between anti-hpGRF-40 antibodies and pGRF-44 made immunoaffinity chromatography a very efficient step for the purification of pGRF from crude extracts. pGRF-44 corresponding to at least 1000 hypothalami, 35-40 µg immunoreactive GRF (irGRF), was quantitatively retained by the column. A typical isolation of this prepurified pGRF-44 is illustrated in Fig. 1. Gel filtration (Fig. 1A) yielded one major irGRF zone which, upon semipreparative HPLC (Fig. 1B), was separated into two irGRF fractions. Rechromatography of the major fraction in an analytical reverse phase HPLC system with different solute selectivity (Fig. 1C) yielded two irGRF peaks, each corresponding to a distinct UV-light-absorbing peak of peptide material. The larger of the two peaks was subjected to structural characterization and represented 3.5 nmol of pGRF-44 per 1000 hypothalami. The chemical nature of the early eluting irGRFs (Figs. 1B and 1C) is not known; it is conceivable that they represent degradation products similar to those found in a human pancreas tumor (1) and in human hypothalamus (10) or are oxidized forms of pGRFs. The amino acid composition of the major pGRF (Table I) suggested that this molecule was structur-

Table I. AMINO ACID COMPOSITIONS of pGRF-44 and ITS CYANOGEN BROMIDE CARBOXYL TERMINAL DIGESTION FRAGMENT*

Amino Acid	pGRF-44. (n=4)		pGRF-44(28-44) (n=1)	
Asx	4.19 ± .08	(4)**	1.31	(1)**
Thr	1.26 ± .07	(1)	0.14	
Ser	2.77 ± .11	(3)	0.93	(1)
Glx	7.75 ± .04	(8)	5.69	(6)
Gly	3.13 ± .27	(3)	1.74	(2)
Ala	4.03 ± .07	(4)	1.04	(1)
Val	1.75 ± .08	(2)	0.88	(1)
Met	1.11 ± .04	(1)	0	
Ile	1.96 ± .09	(2)	0	
Leu	4.90 ± .04	(5)	1.09	(1)
Tyr	1.93 ± .04	(2)	0	
Phe	1.02 ± .04	(1)	0	
His	0		0	
Trp	0		0	
Lys	2.22 ± .50	(2)	0	
Arg	5.98 ± .07	(6)	4.2	(4)
Cya***	0		n.d.	
Pro	0		n.d	

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

** Values in parentheses were deduced from sequence analyses of pGRF-44 and the cyanogen bromide digestion fragment pGRF-44(28-44).

*** Cysteine determined as cysteic acid.

n.d. = not determined

ally very similar to the 44 amino acid human pancreatic growth hormone releasing peptide, hpGRF-44, previously characterized (1). The primary structure of pGRF-44 was determined by sequence analyses of the intact peptide and its carboxyl terminal cyanogen bromide digestion fragment. Direct Edman degradation of 2000 pmol of the intact peptide yielded the sequence of the amino terminal 42 amino acids as shown in Table II. Cyanogen bromide cleavage

Table II. SEQUENCE ANALYSIS of pGRF-44 *

Cycle No. (N)	Residue No.	>PhNCS-AA	Yield (pmol)	Carryover from (N-1) (pmol)
1	1	Tyr	402	-
2	2	Ala	258	37.6
3	3	Asp	184	15.4
4	4	Ala	225	17.7
5	5	Ile	216	18.3
6	6	Phe	187	38.1
7	7	Thr	55.0	16.0
8	8	Asn	188	35.5
9	9	Ser	41.6	44.8
10	10	Tyr	131	13.0
11	11	Arg	141	32.5
12	12	Lys	87.9	77.3
13	13	Val	96.6	32.8
14	14	Leu	120	27.3
15	15	Gly	57.0	39.0
16	16	Gln	74.2	37.9
17	17	Leu	103	35.4
18	18	Ser	7.7	49.8
19	19	Ala	58.4	4.4
20	20	Arg	45.5	20.8
21	21	Lys	24.0	42.6
22	22	Leu	45.2	11.0
23	23	Leu	86.0	-
24	24	Gln	33.3	40.4
25	25	Asp	22.6	22.4
26	26	Ile	36.6	17.9
27	27	Met	24.2	15.8
28	28	Ser	6.9	16.2
29	29	Arg	20.7	2.5
30	30	Gln	40.4	23.2
31	31	Gln	49.2	-
32	32	Gly	18.3	41.4
33	33	Glu	17.3	16.8
34	34	Arg	14.4	14.9
35	35	Asn	9.0	16.5
36	36	Gln	39.3	7.5
37	37	Glu	19.1	39.3
38	38	Gln	40.0	18.9
39	39	Gly	19.0	30.0
40	40	Ala	15.8	19.6
41	41	Arg	8.4	10.0
42	42	Val	2.8	8.4
43	43	X	-	2.6
44	44	X	-	-

* Amount applied: 2000 pmol; Initital yield; 13.8%;
Average repetitive yield: 91.4%

of 3300 pmol pGRF-44 at its single methionine residue and subsequent reverse phase chromatographic isolation of the digestion fragments (data not shown) yielded 750 pmol pGRF-44(28-44) as quantitated by amino acid analysis (Table I). The structure of the carboxy terminal cyanogen bromide digestion fragment was established by Edman degradation of 680 pmol pGRF-44(28-44) as shown in Table III. The identification of the carboxyl terminal leucine was made by comparison of residues found in the established sequence, pGRF-44(28-43) (Table III), with the amino acid composition of pGRF-44(28-44).

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 a free carboxyl or an amidated carboxyl-terminus. These studies were performed with two different solvent systems and clearly show co-elution of native pGRF-44 with synthetic pGRF-44-NH_2 and separation from synthetic pGRF-44-OH (Figure 2). The conclusion that pGRF-44 is amidated is further substantiated by potency analysis of pGRF-44-NH_2 and pGRF-44-OH as assayed in rat pituitary cells. As in the case of hpGRF-44 (8) the amidated peptide is about 2 times as potent as the free acid form (relative potencies 74.7% and 35.6% of hpGRF-44-NH_2, respectively) and native pGRF-44 is equally potent as synthetic pGRF-44-NH_2.

Table III. SEQUENCE ANALYSIS of pGRF-44(28-44)*

Cycle No. (N)	Residue No.	>PhNCS-AA	Yield (pmol)	Carryover from (N-1) (pmol)
1	28	Ser	28.8	-
2	29	Arg	61.1	2.2
3	30	Gln	64.3	5.7
4	31	Gln	91.3	-
5	32	Gly	32.1	15.2
6	33	Glu	28.6	12.6
7	34	Arg	37.1	11.1
8	35	Asn	29.5	10.4
9	36	Gln	37.6	11.0
10	37	Glu	28.2	15.2
11	38	Gln	25.7	14.7
12	39	Gly	12.1	10.1
13	40	Ala	16.2	11.1
14	41	Arg	23.0	7.4
15	42	Val	10.2	8.4
16	43	Arg	14.4	6.0
17	44	X	-	8.8

* Amount applied: 680 pmol; Initial yield: 17.3%; Average repetitive yield: 84.3%

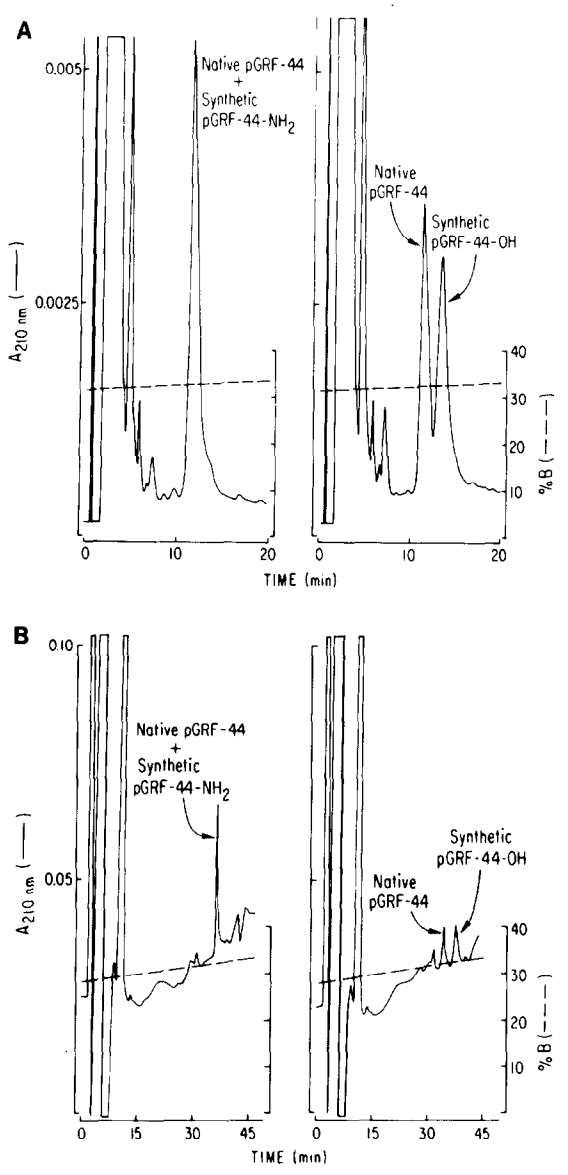

Figure 2. Reverse phase liquid chromatography of native pGRF-44 and synthetic replicates containing either a free acid or an amidated carboxyl terminus. A) Solvent A, 0.25 M TEAP, pH 3.0; Solvent B, 20% 0.25 M TEAP, pH 3.0 and 80% acetonitrile. Elution at 1.5 ml/min was performed with a 20 min linear gradient from 31% to 33% B. 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. All chromatography runs were performed at room temperature with 200 pmol quantites of each peptide.

Preliminary evaluation of the in vitro bioactivity of both the isolated pGRF-44 and its synthetic replicate indicates that the molecule has the full intrinsic activity and 60-82% of the potency of hpGRF-44 (data not shown).

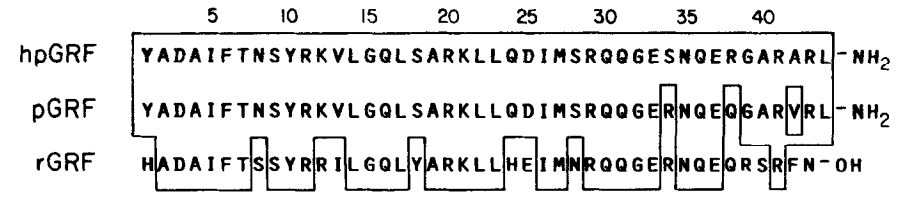

Figure 3. Primary structures of hpGRF-44, pGRF-44 and rGRF-43. Sequence differences from the human structure are outlined.

We have reported here the isolation and the complete structural characterization of a 44 amino acid porcine hypothalamic peptide with high intrinsic growth hormone releasing activity. The structure is very similar to that of hpGRF-44 (3 amino acid substitutions), a growth hormone releasing peptide isolated from a human pancreatic tumor (2) and also found to be present in human hypothalamus (11). Both human and porcine GRF are peptides that contain 44 amino acids and are amidated at their carboxyl termini. In contrast, rat hypothalamic GRF characterized by Spiess et al. (7) contains only 43 amino acids and is not amidated (Fig. 3) Although rGRF-43 is structurally rather different from other GRFs (14 amino acid substitutions as compared to hpGRF-44), the absence of an amidated forty-fourth amino acid in the rat structure is unexpected. The possibility exists that rGRF-43 is a product of limited *in vivo* or *in vitro* degradation and that an amidated rGRF-44 also occurs in the rat hypothalamus. This view is supported by our finding multiple forms of GRF, some of which are carboxyl terminally degraded, in extracts of most GRF-containing tissues (1,11 and this report) including rat hypothalamus (unpublished observations).

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