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CHARACTERIZATION OF A 40 RESIDUE PEPTIDE FROM A HUMAN PANCREATIC TUMOR WITH GROWTH HORMONE RELEASING ACTIVITY

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Summary. Structural characterization of a 40 amino acid peptide with high intrinsic growth hormone releasing activity isolated from a human pancreatic tumor which had caused acromegaly was accomplished by gas phase sequence analyses of the intact peptide and its carboxy terminal cyanogen bromide digestion fragment. High pressure liquid chromatography of the native peptide and synthetic replicates showed that the molecule possessed a free acid rather than an amidated carboxy 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-Ser-Asn-Gln-Glu-Arg-Gly-Ala-OH using 1.8 nmoles of material. The structural identity of this material with a previously characterized fragment of a larger growth hormone releasing peptide isolated from a different human tumor is discussed.

We have recently isolated and characterized a 44 amino acid peptide (hpGRF-44) with high intrinsic growth hormone releasing activity (1) isolated from a pancreatic tumor which had caused acromegaly in a 55 year old patient (Mr. W., Lyon, France). From the same tissue we have also characterized two probable proteolytic degradation products of hpGRF-44 containing the amino terminal 40 (hpGRF-40) and 37 (hpGRF-37) amino acid sequence of the parent molecule and possessing lower specific bioactivities, *in vitro*. The carboxy terminus of hpGRF-44 is amidated while the carboxy termini of hpGRF-40 and hpGRF-37 were shown to be free acids.

One of us (MT) has obtained yet another human pancreatic tumor from an acromegalic patient (Ms. G., Charlottesville, VA) who presented no indications of pituitary abnormality other than somatotroph hyperplasia. From the small fraction of this tumor

ABBREVIATIONS:
hpGRF-44, hpGRF-40 and hpGRF-37: human pancreatic growth hormone releasing factors composed of 44, 40 and 37 amino acids, respectively.

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tissue available to us we have isolated a 40 amino acid peptide which is active both *in vitro* and *in vivo* in stimulating the release of growth hormone. No larger molecular forms possessing growth hormone releasing activity were present in this tissue extract. We describe herein the complete structural characterization of the 40 amino acid molecule.

MATERIALS & METHODS

Materials. A 55 g pancreatic tumor was surgically removed from an acromegalic patient (Ms. G, Charlottesville, VA) who did not have a pituitary adenoma. The five grams of tissue available to us were extracted and a peptide with high intrinsic growth hormone releasing activity was isolated (manuscript in preparation).

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, benzene, ethyl acetate, n-butyl chloride (Burdick & Jackson) and methanol (Matheson, Coleman & Bell) were purified and prepared as described (2) as were two of the four reagents, trifluoroacetic acid (Fluka & Pierce) and 1N HCl in methanol. The 15% phenylisothiocyanate/heptane and 25% trimethylamine/water reagents were obtained from Applied Biosystems.

Cyanogen bromide digestion. One nmole of the peptide in 0.95 ml of 0.5% heptafluorobutyric acid, 43.9% acetonitrile was rapidly dried in a 17 x 100mm polypropylene tube using a Savant vacuum centrifuge. Upon dissolution of the peptide with 100 µl 70% formic acid, a small crystal of cyanogen bromide was added and the reaction mixture capped and vortexed. After incubation for 16 hours in the dark, the cyanogen bromide-generated peptide fragments were purified with an Altex Model 322 high pressure liquid chromatography system equipped with a Brownlee RP-18 guard column (10 µ m; 4.6 x 30mm), a Brownlee RP-18 analytical column (5 µ m; 4.6 x 250mm) and a modified pyridine formate/n-propanol system (3) consisting of 1% pyridine, 1.5% formate in solvents A and B, with B also containing 60% n-propanol.

Amino acid analyses. Peptides were hydrolyzed in sealed evacuated ignition tubes containing five µl 6N HCl and 7% thioglycollic acid for 24 hours at 110°C (4). Amino acid analyses of 5-25 pmoles of peptide hydrolyzates were performed with a Liquimat III amino acid analyzer (Kontron, Zurich, Switzerland) equipped with a proline conversion detection system (5).

Edman degradation. The sequential degradation of peptides was performed with an Applied Biosystems Model 470A gas phase sequencer as described (2) with several modifications. The dipeptide, Phe-Leu, was used instead of Gly-Gly to precycle the Polybrene (Aldrich). Longer coupling times (1200 sec) in all cycles and a longer cleavage time (1665 sec) in the first cycle were used to enhance the repetitive and initial yields, respectively. Finally, an extensive methanol wash (180 sec, 3.8 ml) was used to clean the conversion flask after each cycle and thus reduce cycle to cycle carryover. The phenylthiohydantoin amino acid derivatives were unambiguously identified by high pressure liquid chromatography as described (6).

High pressure liquid chromatography comparison of native hpGRF-40 and synthetic replicates. Synthetic replicates of hpGRF-40 were synthesized in two forms, i.e., with a free acid at the carboxy terminus and with an amidated carboxy terminus using solid phase methodology as described (7). Reverse phase high pressure liquid chromatography was employed to effect separation between the free acid and amidated forms of the peptide and, in turn, to ascertain with which form the native peptide would co-elute. This was accomplished with an Altex Model 322 high pressure liquid chromatography system, a Brownlee RP-18 (10 µ m; 4.6 x 30mm) guard column, an Altex Ultrasphere ODS (5 µ m; 4.6 x 250 mm) analytical column and triethylammonium phosphate/acetonitrile buffers (8). Chromatography conditions are described in Figure 2.

TABLE I

Amino Acid Compositions of hpGRF-40 and Its Cyanogen Bromide Digestion Fragments

Amino Acid	hpGRF-40 (n=6)		hpGRF-40(1-27) (n=2)		hpGRF-40(28-40) (n=2)	
Asx	4.13 ± 0.38	(4)[a]	3.20 ± 0.12	(3)[a]	1.02 ± 0.06	(1)[a]
Thr	0.93 ± 0.19	(1)	0.92 ± 0.04	(1)		
Ser	3.40 ± 0.13	(4)	1.84 ± 0.13	(2)	1.67 ± 0.04	(2)
Glx + Hse	7.14 ± 0.44	(7)	3.02 ± 0.13	(2+1)	5.19 ± 0	(5)
Gly	3.08 ± 0.07	(3)	0.86 ± 0.29	(1)	1.89 ± 0.02	(2)
Ala	4.02 ± 0.06	(4)	3.21 ± 0.01	(3)	1.06 ± 0.06	(1)
Val	1.10 ± 0.07	(1)	0.91 ± 0.01	(1)		
Met	1.01 ± 0.22	(1)		(1)		
Ile	1.76 ± 0.10	(2)	1.77 ± 0.03	(2)		
Leu	4.33 ± 0.26	(4)	4.24 ± 0.11	(4)		
Tyr	1.95 ± 0.17	(2)	1.68 ± 0.09	(2)		
Phe	1.04 ± 0.15	(1)	0.60 ± 0.25	(1)		
His	0.18 ± 0.16	(0)				
Trp	0	(0)				
Lys	2.59 ± 0.15	(2)	2.39 ± 0.15	(2)		
Arg	4.10 ± 0.29	(4)	2.27 ± 0.07	(2)	2.14 ± 0.02	(2)
Cya	0	(0)				
Pro	0.35 ± 0.27	(0)				
Total Peptide Recovered (pmol)	1800		54.5		132.5	

[a] Values in parentheses were deduced from sequence analyses of intact hpGRF-40 and the cyanogen bromide digestion fragment hpGRF-40(28-40)

RESULTS

Edman degradation of hpGRF-40 and its cyanogen bromide digestion fragments. The amino acid composition of hpGRF-40 (Table I) suggested that this molecule was structurally very similar to a 40 amino acid hpGRF peptide previously characterized (1). The primary structure for hpGRF-40 was determined by sequence analyses of the intact peptide and its carboxy terminal cyanogen bromide digestion fragment. The structure of hpGRF-40(1-33) was established by direct Edman degradation of 500 pmoles of the intact peptide as shown in Table II. Cyanogen bromide cleavage of 1 nmole hpGRF-40 at its single methionine residue and subsequent high pressure liquid chromatographic isolation of the digestion fragments (Figure 1) yielded 54 pmoles hpGRF-40(1-27) and 132 pmoles hpGRF-40(28-40) by quantitative amino acid analyses (Table I). The structure of the carboxy terminal cyanogen bromide digestion fragment was established by Edman degradation of 90 pmoles hpGRF-40(28-40) as shown in Table III. Hence, the identities of all the amino acids in hpGRF-40 were established by direct identification and a five residue overlap in

TABLE II

SEQUENCE ANALYSIS OF hpGRF-40

Amount Applied: 500 pmol
Initial Yield: 40.0% Average Repetitive Yield: 91.4%

Cycle No. (N)	Residue No.	>PhNCS-AA	Yield (pmol)	Carryover from (N-1) (pmol)
1	1	Tyr	341	-
2	2	Ala	219	10.4
3	3	Asp	124	11.6
4	4	Ala	209	19.1
5	5	Ile	160	40.5
6	6	Phe	135	10.5
7	7	Thr	43.9	9.5
8	8	Asn	89.7	9.9
9	9	Ser	35.2	12.7
10	10	Tyr	78.5	8.6
11	11	Arg	108	17.5
12	12	Lys	92.9	38.9
13	13	Val	96.1	18.4
14	14	Leu	85.2	24.9
15	15	Gly	21.6	33.7
16	16	Gln	51.7	16.7
17	17	Leu	46.5	18.1
18	18	Ser	1.5	26.4
19	19	Ala	37.6	0
20	20	Arg	31.6	20.3
21	21	Lys	26.9	18.4
22	22	Leu	35.3	13.5
23	23	Leu	39.3	-
24	24	Gln	31.4	28.5
25	25	Asp	15.7	26.1
26	26	Ile	15.7	7.9
27	27	Met	10.2	10.1
28	28	Ser	2.1	0.9
29	29	Arg	21.7	1.0
30	30	Gln	17.8	12.9
31	31	Gln	25.1	-
32	32	Gly	9.5	19.3
33	33	Glu	13.9	6.9
34	34	X	-	10.3
35	35	X	-	-
36	36	X	-	-
37	37	X	-	-
38	38	X	-	-
39	39	X	-	-
40	40	X	-	-

sequence information between hpGRF-40(1-33) and hpGRF-40(28-40) was obtained from Edman degradation data to confirm the complete structure.

Determination of the carboxy terminus of hpGRF-40. The nature of the carboxy terminus was established by high pressure liquid chromatography studies in which the native peptide was co-chromatographed with synthetic replicates possessing a free carboxyl or an amidated carboxy terminus. These studies, illustrated in Figure 2, clearly show co-elution of native hpGRF-40 with its synthetic replicate possessing a free acid at

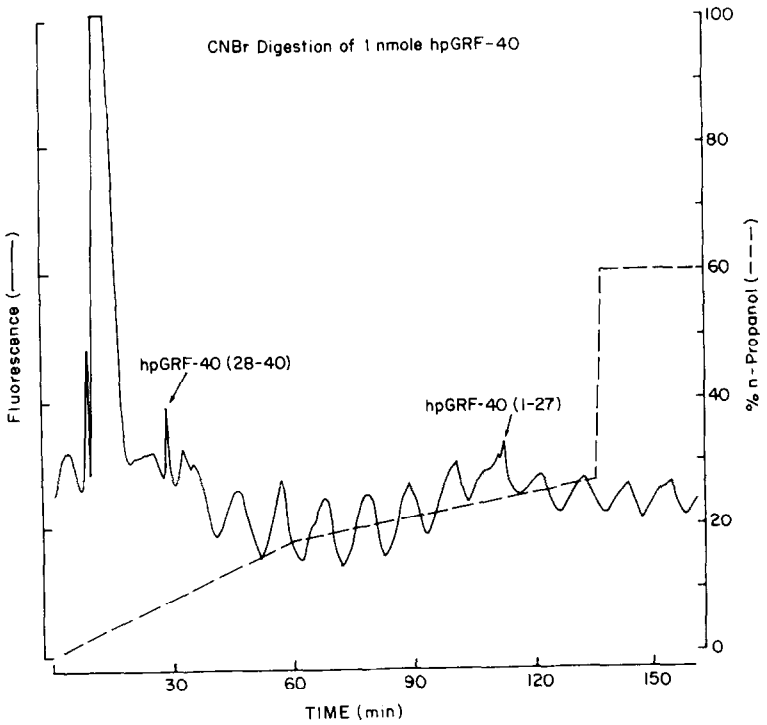

Figure 1. Reverse phase liquid chromatography of a cyanogen bromide digest of hpGRF-40. Digestion and chromatography protocols were as described in Methods. Gradient elutions were at 0.6 ml/min and room temperature. The column effluent was collected in 1.1 ml fractions and monitored with an automatic fluorescamine detection system (9).

its carboxy terminus and separation from its synthetic replicate possessing an amidated carboxy terminus.

DISCUSSION

We have reported here the complete structural characterization of a 40 amino acid peptide with high intrinsic growth hormone releasing activity isolated from a human pancreatic carcinoma. The structure of this 40 residue peptide is identical with that of a fragment obtained from a completely different tumor (1) and suggests that this molecule may also be a proteolytic degradation product of a larger parent structure. The absence, however, of such a larger peptide with growth hormone releasing activity in this tumor may reflect differential proteolytic degradation in different tumor tissues. The small quantities of peptide (1.8 nmoles) available for sequence analysis necessitated the utilization of a reliable and exquisitely sensitive characterization methodology. The importance of the gas phase sequencing technique can not be overemphasized as success-

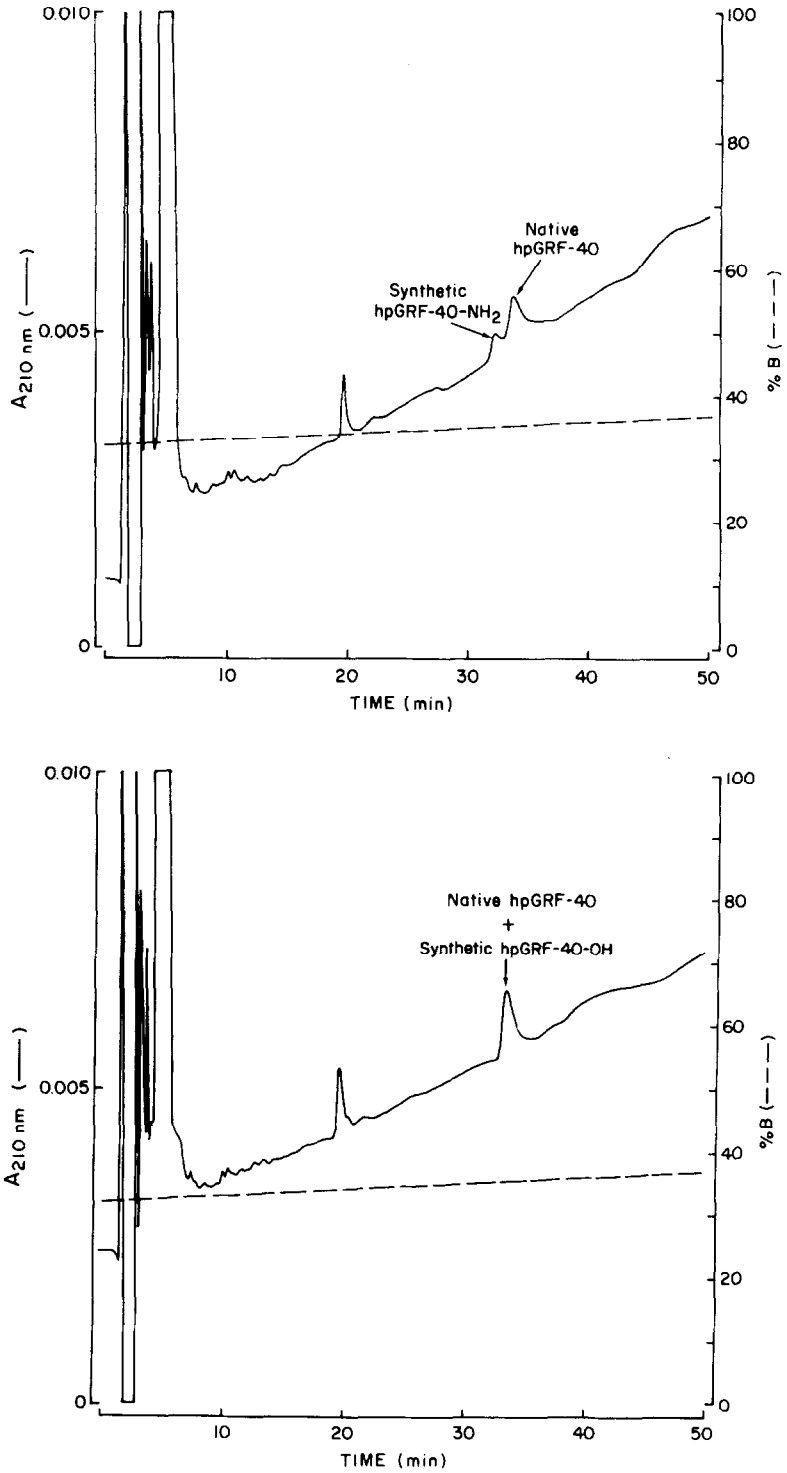

Figure 2. Reverse phase liquid chromatography of native hpGRF-40 and its synthetic replicates containing either a free acid or an amidated carboxy terminus. Solvent A, 0.25N triethylammonium phosphate, pH 3.0; Solvent B, 20% 0.25N triethylammonium phosphate, pH 3.0 and 80% acetonitrile. Elution of 25-50 pmoles of each of the peptides at 1.5 ml/min and room temperature employed a 50 min linear gradient from 32% to 37% B. Remaining chromatography conditions are described in Methods.

TABLE III

SEQUENCE ANALYSIS OF THE CARBOXY TERMINAL

CYANOGEN BROMIDE DIGESTION FRAMENT hpGRF-40(28-40)

Amount Applied: 90 pmol

Initial Yield: 27.1% Average Repetitive Yield: 85.0%

Cycle No. (N)	Residue No.	>PhNCS-AA	Yield (pmol)	Carryover from (N-1) (pmol)
1	28	Ser	12.1	-
2	29	Arg	22.4	0
3	30	Gln	22.1	0
4	31	Gln	11.5	-
5	32	Gly	26.8	0
6	33	Glu	23.4	2.8
7	34	Ser	6.4	0
8	35	Asn	23.8	0
9	36	Gln	1.4	0
10	37	Glu	15.0	0
11	38	Arg	5.2	0
12	39	Gly	12.2	0
13	40	Ala	2.0	0

ful sequence analyses of 90-500 pmole quantities of peptides were responsible for bringing this work to fruition.

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