PRIMARY STRUCTURE OF SOMATOMEDIN B

A growth hormone-dependent serum factor with protease inhibiting activity

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1. Introduction

The in vivo action of growth hormone (somatotropin) is presumed to be mediated by polypeptides, the so called somatomedins, non-covalently bound to carrier proteins found in plasma [1,2]. During the isolation of sulfation factor, or somatomedin A, fractions were also examined for stimulation of DNA synthesis in glial cells in culture. A different type of activity to the sulfation factor, which also appeared to be growth hormone-dependent was found [3]. This was termed somatomedin B (SM-B) [4], later isolated and purified to homogeneity [5] and shown to be a protein, mol. wt 5000, cross-linked by four disulfide bridges with N-terminal aspartic acid. Four rather similar components were found, differing by electrophoretic mobility. It is possible that components 3 and 4 are homologues; the amino acid composition suggests a slight difference in certain residues. Antiserum developed to the impure compound [6] and later here with the pure protein showed a relationship to growth hormone status. Serum levels were found to be higher in acromegaly, and in pituitary dwarfs at the lower level of the normal range [7]. High levels were also found at delivery and during oestrogen treatment [8]. The primary structure has been determined and shows that SM-B is not only related structurally to small trypsin inhibitors, but also has an 'active site' region, and inhibits trypsin, but not plasmin, thrombin or kallikrein.

2. Material and methods

2.1. *Somatomedin B*

This was isolated from human plasma and corresponds to fraction 1 [5]. The reduced and alkylated derivative (RCM) was prepared essentially as in [9].

Iodo[^{14}C]acetic acid (New England Nuclear) was used essentially as in [10] to allow for ready localization of peptides.

2.2. *Enzymatic digestions*

These were performed on the RCM derivative essentially as in [11] using the following enzymes: trypsin (Worthington), chymotrypsin (Worthington), carboxypeptidase A (Worthington), carboxypeptidase B (Worthington), and staphylococcal protease II [12] (personal gift).

The staphyloccal protease digestion was performed in 0.1 M NH_4HCO_3 at an enzyme/substrate ratio of 1:100, at room temperature for 24 h, followed by an immediate gel filtration in acid medium.

2.3. *Separation of peptides*

This was performed essentially as in [11]: an initial gel filtration on Sephadex G-25 (1 X 140 cm) in 0.02 N HCl; fractions were read spectrophotometrically at 230 nm, at 570 nm (ninhydrin) after alkaline hydrolysis, or else by radioactivity in aliquots measured in a liquid scintillation counter. The subsequent stage was usually column electrophoresis on cellulose,

at pH 5.0, in 0.05 M pyridine acetate buffer (column size 1 X 100 cm or 0.75 X 50 cm [11]).

2.4. *Analysis of peptides*

2.4.1. Amino acid analysis

Peptide, < 10 nmol, was hydrolyzed in 6 N HCl and analyzed on a Durrum D-500 analyzer by Dr D. Eaker, Institute of Biochemistry, Uppsala.

2.4.2. Edman degradation

This was performed in principle according to the direct Edman technique as in [11,13] with the modifications:

(i) The number of benzene washes was reduced to two or less.

(ii) The thin-layer chromatography was performed on 10 X 10 cm plates instead of 20 X 20 cm.

This allowed shorter running times, sharper spots and greater sensitivity.

3. Results

The amino acid composition of the native substance and the relevant peptides are shown in table 1.

Chromatographic and electrophoretic peptide separations are shown in fig.1,2. Figure 1 shows the separation of tryptic peptides by gel filtration on Sephadex G-25; fraction 1 was further separated by column electrophoresis into 2 main components, yielding the peptide Tryp-1. Figure 2 shows the separation of peptides from the digestion with S-protease, fractions B and C from gel filtration could be further separated by electrophoresis. The chymotryptic peptides were also separated in a similar manner, Chy-2 was obtained pure from the gel filtration while Chy-1 was obtained from additional electrophoresis. As can be seen from table 1, all amino acid compositions are close to integral values and indicate homogeneity.

Edman degradation of the intact RCM derivative identified residues 1–16, and placed the unique Phe at position 13 and one of the two Val residues at position 15 (fig.1). Carboxypeptidase A digest of this same material gave Val–Thr as residues 43–44. No further amino acids were released with carboxypeptidase B. Tryptic digestion gave a peptide of 26 amino acids (Tryp-1) containing 6 residues of cysteine, one Val and one Pro, suggesting that this was the carboxyl terminal, residues 19–44, and that

Table 1
Somatomedin B – peptides

Residue	SM-B		Tryp-1 19–44		SP-1 12–23		SP-2 24–38		SP-3 39–44		Chy-1 29–35		Chy-2 36–44	
Asp	5.23	5	2.04	2	2.99	3	2.80	3	–		1.00	1	–	
Thr	3.96	4	3.77	3	–		2.35	2	1.02	1	1.02	1	2.06	2
Ser	3.09	3	2.08	2	–		2.16	2	–		0.93	1	–	
Glu	8.12	8	5.02	5	2.19	2	2.05	2	1.06	1	1.12	1	2.25	2
Pro	0.77	1	1.06	1	–		–		0.92	1	–		1.00	1
Gly	2.07	2	–		1.04	1	–		–		–		–	
Ala	1.06	1	1.02	1	–		1.12	1	–		–		0.99	1
Cys	8.00	8	5.50	6	1.74	2	3.20	3	0.96	1	1.75	2	0.87	1
Val	1.83	2	0.97	1	0.95	1	–		1.00	1	–		0.96	1
Leu	0.95	1	1.03	1	–		1.02	1	–		–		–	
Tyr	2.90	3	2.53	3	–		–		–		1.15	1	–	
Phe	0.99	1	–		0.93	1	3.12	3	–		–		–	
Lys	3.83	4	0.98	1	1.99	2	–		1.14	1	–		1.01	1
Arg	1.27	1	–		–		–		–		–		–	
Total		44		26		12		17		6		7		9

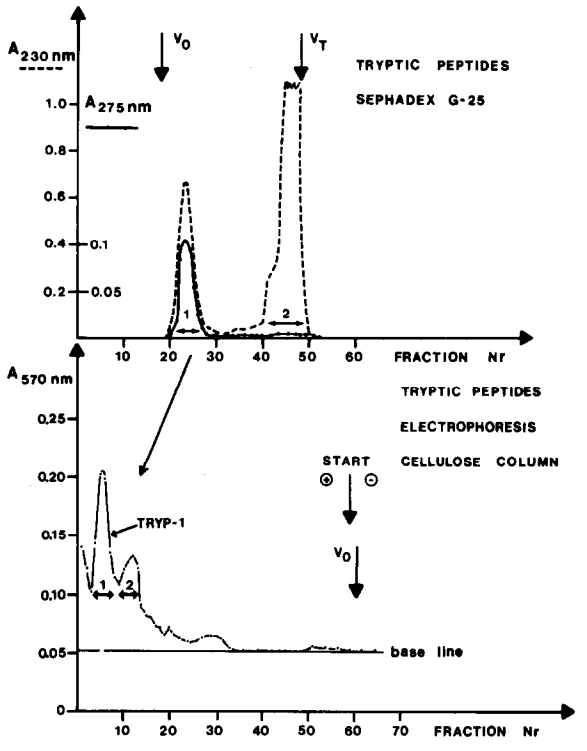

Fig.1. Separation by gel filtration of tryptic peptides (from 100 nmol, 500 μg protein) on Sephadex G-25 (1 × 140 cm) in 0.02 N HCl (2.3 ml fractions). Fraction 1 separated by cellulose column electrophoresis (1 × 99 cm), pH 5.0 (0.05 M pyridine acetate), 18 h, 9.8 mA, 1000 V, migration towards the anode. Fractions, 1 ml, collected.

residue 18 was probably Lys and that a Lys–Pro sequence was present. Nine stages of degradation on this peptide gave residues 19–27 and placed the single Leu at position 24. Digestion with the staphylococcal protease, which splits specifically on the carboxyl side of glutamic acid, gave 5 peptides, identified from the above, and by amino acid compositions, as residues 1–3, 4–11, 12–23 (SP-1), 24–38 (SP-2), 39–44 (SP-3). Edman degradation on SP-1 was performed through 11 stages and free Glu (determined by amino acid analysis) at residue 23 and the overlap 18–19 were confirmed. SP-2 was degraded 11 stages to Cys 34, and with carboxypeptidase A the sequence 35–38 was obtained. Edman degradation of SP-3 for 5 stages allowed the identification of free Thr (by amino acid analysis) at residue 44. The chymotryptic digest gave 2 peptides relevant here, 29–35 (Chy-1) and 36–44 (Chy-2), providing confirmation of the Tyr at residue 35, the overlap 35–36 and the sequence 36–38 by Edman degradation. All of the 44 residues in somatomedin B have therefore been accounted for and the primary structure is shown in fig.3.

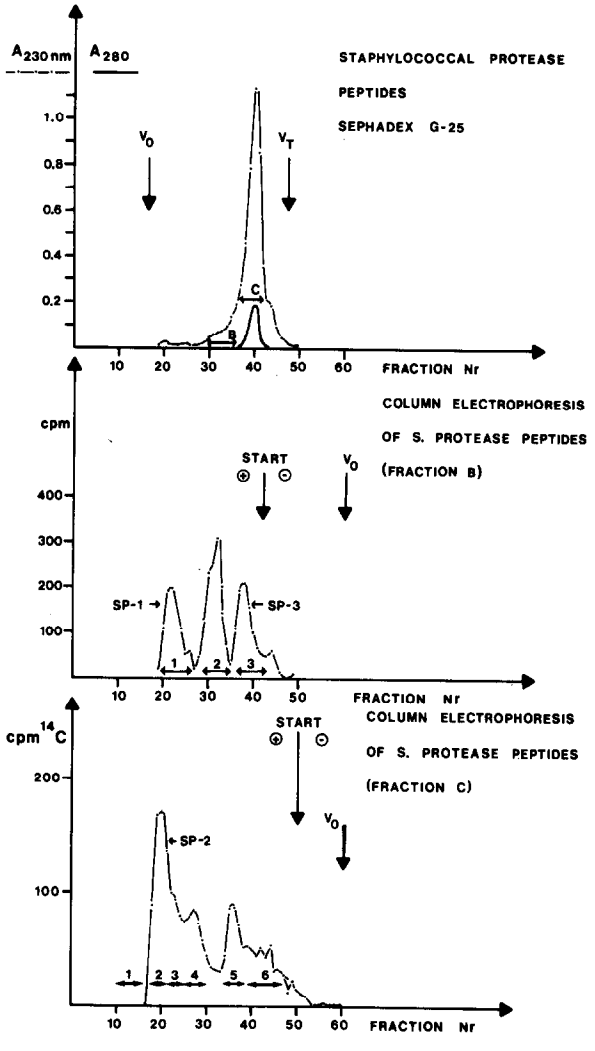

Fig.2. Separation by gel filtration of staphylococcal protease peptides (from 100 nmol, 500 μg protein) on Sephadex G-25 (1 × 140 cm) in 0.02 N HCl (2.3 ml fractions). Fraction B separated by cellulose column electrophoresis (1 × 96 cm) for 15 h, 9 mA and 1000 V, in 0.05 M pyridine acetate, migration towards the anode. Fractions, 1 ml, collected. Fraction C separated under identical conditions.

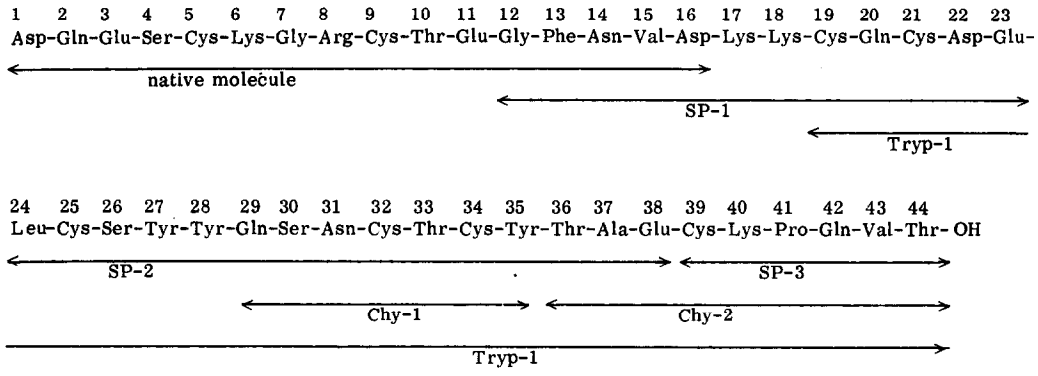

Fig.3. Complete primary structure of somatomedin B.

Results 'SEARCH'

The total number of sequences searched = 1003

The total number of segments of length 25 compared to the test piece = 83941

The constant 2 has been added to the mutation data 1976

				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
The test piece				D	Q	E	S	C	K	G	R	C	T	E	G	F	N	V	D	K	K	C	Q	C	D	E	L	C
Score		Beg.	End																									
1	96	14	38	S	S	K	P	C	(C.	B)	H	(C.	A.	C)	T	K.	S	I	P	P	Q	C	R.	C	T	D	L	R
2	93	13	37	E	Q	G	P	C	K	G	R	I	P	R	Y	F	Y	N	P	A	S	R	M	C	E	S	F	I
3	89	6	30	T	P	D	V	T	S	Q	I	C	A	D	G	H	V	C	Y	T	K	T	W	C	D	N	F	C
4	88	4	28	S	S	K	P	C	C	D	Q	C	A	C	T	K	S	N	P	P	Q	C	R	C	S	D	M	R
5	88	100	124	V	H	D	C	C	Y	G	K	I	Y	W	W	F	P	F	A	K	N	C	Q	C	E	S	P	E
6	87	10	34	S	S	K	P	C	C	D	L	C	M	C	T	A	S	M	P	P	Q	C	H	C	A	D	I	R
7	79	10	34	Y	T	G	P	C	K	A	R	I	I	R	Y	F	Y	N	A	K	S	G	L	C	Q	T	F	V
The test piece				D	Q	E	S	C	K	G	R	C	T	E	G	F	N	V	D	K	K	C	Q	C	D	E	L	C

1 Protease inhibitor – lima bean
2 Protease inhibitor – red sea turtle (fragment)
3 Long neurotoxin 2 – forest cobra
4 Protease inhibitor (Bowman-Birk) – soybean
5 Phospholipase A2 – mamushi
6 Protease inhibitor C-II – soybean
7 Basic trypsin inhibitor (bovine)

Fig.4. Computer alignment of 1st 25 residues of somatomedin B with other sequences.

4. Discussion

The primary structure of somatomedin B shows it to be a unique protein not obviously related to insulin or any of the other known growth factors. The 3 tyrosine residues are clustered in the latter third of the molecule, while 4 of the five basic amino acids are present within the first 18 residues; a Lys–Lys sequence being found at residue 17–18. Of the 13 glutamic and aspartic acid residues, 6 are amidated, resulting in a net negative charge of 2. A computer sequence search of the first 25 residues performed [14] (Program SEARCH) suggested a close relationship to protease inhibitors and phospholipases. The alignments are shown in fig.4. A closer examination of the N-terminal sequence of SM-B revealed in residues 5–8 (Cys–Lys–Gly–Arg) a stretch of sequence almost identical to that found in the basic bovine trypsin inhibitor [15], residues 14–17 (Cys–Lys–Ala–Arg), where the Lys–Ala peptide bond is the cleavage site for trypsin. During our attempts to determine the disulfide pairing of SM-B, we observed that the native molecule was extremely resistant to cleavage by proteolytic enzymes. The protease inhibitory activity of somatomedin B was tested using the chromogenic peptide substrates [16,17] for trypsin, plasmin, kallikrein and thrombin. Only trypsin was specifically inhibited [18]. When iodinated by the lactoperoxidase method SM-B bound poorly or not at all to most membranes [19] except testis. Perhaps the proximity of the tyrosine residues means that addition of the large iodine molecule in some way hinders binding, or else no specific binding site exists. Iodination does not prevent measurement by radioimmunoassay, but the iodinated protein does not appear to recombine with its carrier protein [7]. Radioimmunoassay measurements in serum show a gradual decline in levels with age, a positive correlation with overproduction of GH [20] and during oestrogen treatment [8]. A rise is seen during pregnancy which reaches a maximum at delivery. In vivo studies on hypophysectomized rats studying membrane transport of amino acids as an indirect measure of protein synthesis show that the steady-state level in blood serum of the non-metabolizable amino acid, α-aminoisobutyric acid, is decreased, concomitant with a decrease in blood sugar [21].

Up to now the role and function of somatomedin B have been somewhat enigmatic. A growth hormone-dependent trypsin-like protease inhibitor could have effects on controlled proteolysis during conversion of prohormones, or on the action of the 'true' somatomedins, for example, the regulation of receptor functions or membrane transport. We know that sometomedin B does not compete in the radioreceptor assay for SM-A [22], or have activity on chick fibroblasts or adipocytes [23] but the protein has not been tested in combination with any other somatomedins or growth factors. Eventual activity in the serum carrier protein for somatomedin B has not been investigated. Further experiments on the biological role of SM-B and its carrier protein are in progress.

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