Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5233–5237, July 1990
Biochemistry

Complete amino acid sequence of bovine glia maturation factor β

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Communicated by Hewson Swift, April 30, 1990

ABSTRACT **The protein glia maturation factor β, isolated from bovine brain, has been sequenced by automated Edman degradation and tandem mass spectrometry of overlapped peptide fragments generated by cyanogen bromide cleavage and enzymatic digestion with trypsin, chymotrypsin, and endoproteinases Asp-N and Lys-C. The protein has 141 amino acid residues and possesses no potential N-glycosylation sites. It contains three cysteines (at positions 7, 86, and 95), three methionines (at positions 33, 101, and 102), and one tryptophan (at position 132). The blocked amino terminus as determined by tandem mass spectrometry is an N-acetylated serine. The carboxyl terminus is a histidine. To our knowledge, the sequence shows no significant homology with other sequenced proteins. The molecular weight calculated from the sequence information is 16,582.**

Glia maturation factor β (GMF-β), first detected in this laboratory (1–3), has been purified to homogeneity from the bovine brain (4). It is a 17-kDa acidic (pI = 4.9) protein distinct from other growth-regulating molecules isolated from the nervous system. Unlike the fibroblast growth factors whose functions are primarily mitogenic, GMF-β exhibits a strong antiproliferative effect on neuronal and glial tumor cells (4, 5). GMF-β is found in the brains of all vertebrates examined (6, 7). The level is highest in embryos, although its presence persists throughout life (7). In the central nervous system, GMF-β is localized to astrocytes (7) and may mediate both autocrine function and glial–neuron interaction. Repeated application of GMF-β into the injured cerebral cortex increases the number of large neurofilament-rich neurons (8). In the peripheral nervous system, GMF-β is inducible in Schwann cells in response to loss of axonal contact (9). Thus, GMF-β appears to play a role in differentiation, regeneration, and maintaining the integrity of the normal mature nervous system. In this paper we present the complete amino acid sequence of bovine GMF-β obtained by automated Edman degradation and tandem mass spectrometry.§

MATERIALS AND METHODS

Preparation of Bovine GMF-β. GMF-β was isolated from bovine brains by the method described (4).

Reduction and Alkylation of GMF-β. GMF-β was reduced for 2 hr at 37°C in 100 μl of 7 M guanidine hydrochloride/0.5 M Tris·HCl, pH 8.2/2 mM EDTA with dithiothreitol added to a final concentration of 30 mM. Samples were subsequently alkylated for 2 hr at room temperature under argon by addition of either 4-vinylpyridine or iodoacetic acid, as indicated, to a concentration of 70 mM. The sample was used directly for Asp-N digestion without further treatment. For other enzyme digestion and cyanogen bromide cleavage, the samples were first freed of the excess reagents by precipitation with 95% ethanol for 24 hr at −20°C or by desalting over a Vydac C_4 column using a linear gradient of 5–80% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid.

Narrow-Bore Reverse-Phase HPLC Separation of Peptides. Peptide mixtures resulting from cleavage of GMF-β were chromatographed on a Hewlett–Packard 1090 HPLC system equipped with a model 1040 diode array detector, using a reverse-phase Vydac 2.1 mm × 150 mm C_{18} column (The Nest Group, Southboro, MA), except for tryptic peptides where a C_8 column was used. The gradient employed was a modification of that described by Stone *et al.* (10). Briefly, where buffer A was 0.06% trifluoroacetic acid/H_2O and buffer B was 0.055% trifluoroacetic acid/acetonitrile, a gradient of 5% buffer B at 0 min, 33% buffer B at 63 min, 60% buffer B at 95 min, and 80% buffer B at 105 min, with a flow rate of 150 μl/min was used. Chromatographic data at 210 nm, 254 nm, and 277 nm and UV spectra from 209 to 321 nm of each peak were acquired. Fractions were manually collected and immediately stored without drying at −20°C in preparation for microsequencing.

Amino Acid Composition Analysis. Samples for amino acid analysis were hydrolyzed with 6 M HCl at 110°C for 20 hr under argon. The hydrolysates were spotted on the glass frits of an Applied Biosystems model 420A derivatizer for automated derivatization with phenyl isothiocyanate and subsequent analysis by on-line HPLC (Applied Biosystems model 130A).

Microsequencing of Peptides. Peptide samples for sequence analysis were applied to a Polybrene precycled glass fiber filter and placed in the reaction cartridge of an Applied Biosystems model 477A protein sequencer. The samples were subjected to automated Edman degradation using the standard program NORMAL-1, which was modified for faster cycle time (37 min) by decreasing dry-down times and increasing reaction cartridge temperature to 53°C during coupling (M. Kochesperger, Applied Biosystems; personal communication). The resultant phenylthiohydantoin amino acid fractions were identified by online HPLC (Applied Biosystems model 120A) and manual interpretation of the chromatographic data.

Anhydrotrypsin Affinity Chromatography. Anhydrotrypsin affinity column chromatography was conducted as described by Kumazaki *et al.* (11). Briefly, a tryptic digest of GMF-β was passed through a column of immobilized anhydrotrypsin (Pierce). The carboxyl-terminal peptide was recovered from the flow-through fraction for sequence analysis.

Fast Atom Bombardment Mass Spectrometry (FABMS). FABMS of peptides was carried out in the first (MS-1) of two mass spectrometers of a tandem high-resolution mass spectrometer (JEOL HX110/HX110) at an accelerating voltage of 10 kV and a resolution of 1:2600. Single scans were acquired at a rate to scan from m/z 0 to m/z 6000 in 2.5 min and with

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Abbreviations: GMF, glia maturation factor; FABMS, fast atom bombardment mass spectrometry; CID, collision-induced dissociation.

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§The sequence reported in this paper has been deposited in the Protein Identification Resource data base (accession no. A33066).

filtering at 100–300 Hz. For calibration, $(CsI)_nCs^+$ cluster ions were used. The JEOL Cs^+ gun was operated at 26 kV.

Tandem Mass Spectrometry. Tandem mass spectrometry was carried out by using all four sectors of the JEOL HX110/HX110, an instrument of $E_1B_1E_2B_2$ configuration. Collision-induced dissociation (CID) of protonated peptide molecules, selected with MS-1, took place in the field-free region after B_1, thus operating both MS-1 and MS-2 as double-focusing instruments. The collision-cell potential was held at 3.0 kV; the ion-collision energies were at 7.0 keV. Helium, the collision gas, was introduced at a pressure sufficient to reduce the precursor ion signal by 65–70%. CID mass spectra were recorded with filtering at 100 Hz at a rate to scan from m/z 40 to m/z 1900 in 54 sec; two scans were acquired and summed for each spectrum. MS-1 was operated at a resolution that permitted the transmission of only the ^{12}C species of the protonated peptide molecule. MS-2 was operated at a resolution of 1:1000 and was calibrated with a mixture of CsI, NaI, KI, RbI, and LiCl.

Sample Introduction and Data Acquisition for Mass Spectrometry. A 5-μl syringe was used to deposit 0.5 μl of matrix, with the peptides dissolved therein, on the FABMS probe. A 1:1 (vol/vol) mixture of glycerol and 3-nitrobenzyl alcohol was used as the FAB matrix. All mass spectra are raw profile data and were recorded with a JEOL model DA5000 data system. Further information on mass spectrometry is found in refs. 12–15.

RESULTS

The sequence of bovine GMF-β is summarized in Fig. 1. The final result was obtained by aligning overlapped peptide fragments from cyanogen bromide cleavage and from digestions with trypsin, chymotrypsin, Asp-N, and Lys-C, the latter in combination with mass spectrometry. Aside from N-acetylation of the first residue, there are no unusual features. No asparagine glycosylation sequences (Asn-Xaa-Ser/Thr) are present. The total residues add up to 141 and the composition agrees well with that obtained by direct amino acid analysis of the protein (Table 1). The molecular weight calculated from the sequence is 16,582 (not including the N-acetyl group). Details of sequence determination are presented below.

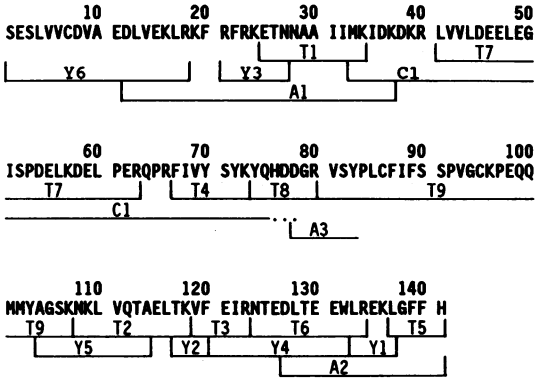

FIG. 1. Primary structure of bovine GMF-β constructed by alignment of overlapped fragments after partial degradation. The single-letter amino acid code is used. All peptides were sequenced by Edman degradation with a microsequencer. Each continuous line starting with a bar represents one peptide fragment. A line ending with a bar indicates that the peptide was sequenced to the end. A line ending with three dots indicates that the peptide was sequenced to the point where no more meaningful information was obtainable, although additional residues existed. A line ending with neither indicates that the cycle was intentionally terminated, although further sequencing was possible. The sources of the peptides are coded as follows: T, tryptic digestion; C, cyanogen bromide cleavage; Y, chymotryptic digestion; A, Asp-N digestion. Numbers after the peptide codes correspond to peaks in the HPLC elution profiles (see Fig. 2). Only peptide fragments yielding unambiguous residue assignments were used in the construct. Redundant confirmatory fragments are omitted for the sake of clarity. No unmatched peptides were observed.

Tryptic Digestion. Repeated attempts to sequence natural bovine GMF-β did not yield meaningful residues, indicating that the protein is amino-terminally blocked. Therefore, the protein was digested with endopeptidases to generate peptides for sequencing. With tryptic digestion, 28 peptides were generated (Fig. 2*A*); the sequences of 9 peptides are shown in Fig. 1. Tryptic digestion identified 106 residues in scattered fragments, amounting to 75% of the total estimated residues.

Cyanogen Bromide Cleavage. The result of cyanogen bromide cleavage is shown in Fig. 2*B*. The separation on HPLC was less than ideal. However, one peptide peak (C1) yielded the sequence from position 33 to position 75, which overlapped peptides T1, T7, T4, and T8. This established linearity from position 25 to position 80 (Fig. 1).

Chymotryptic Digestion. Treatment with chymotrypsin generated many peaks (Fig. 2*C*). Of these, five peaks (peaks 1–5) were sequenced and aligned in Fig. 1, extending the linearity from position 21 to position 80 and from position 81 to position 141. On a repeat chymotryptic digestion and subsequent HPLC separation, peak 6 (Fig. 2*C*) became a doublet (data not shown), differing by one additional carboxyl-terminal amino acid (arginine) between the two members. The sequence of the larger peptide was presented as Y6 in Fig. 1. It occupied positions 1–18 in the final scheme (see *Discussion*). The amino acid composition of this fragment, which turned out to be an amino-terminal peptide, is shown in Table 2.

Endoproteinase Asp-N Digestion. Fig. 2*D* shows the result of digestion with endoproteinase Asp-N. Three of the peaks separated by HPLC were sequenced and presented in Fig. 1.

Table 1. Amino acid composition of GMF-β

Amino acid	Amino acid residues, mol/mol of protein	
	Sequence	Amino acid analysis*
Asx	14	14
Glx	22	24
Ser	8	8
Gly	5	5
His	2	2
Arg	9	8
Thr	5	6
Ala	5	6
Pro	6	6
Tyr	5	5
Val	11	10
Ile	7	7
Leu	14	12
Phe	8	8
Lys	13	12
Cys	3	3
Met	3	3
Trp	1	1
Total residues, no.	141	140
M_r (calcd)	16,554†	16,375

*Data from ref. 4.

†Note that this value is slightly different from that presented in the text because, for ease of comparison, the value in this table was calculated according to the convention for amino acid analysis—i.e., by using round numbers for residue mass and treating asparagine and glutamine as aspartic acid and glutamic acid, respectively.

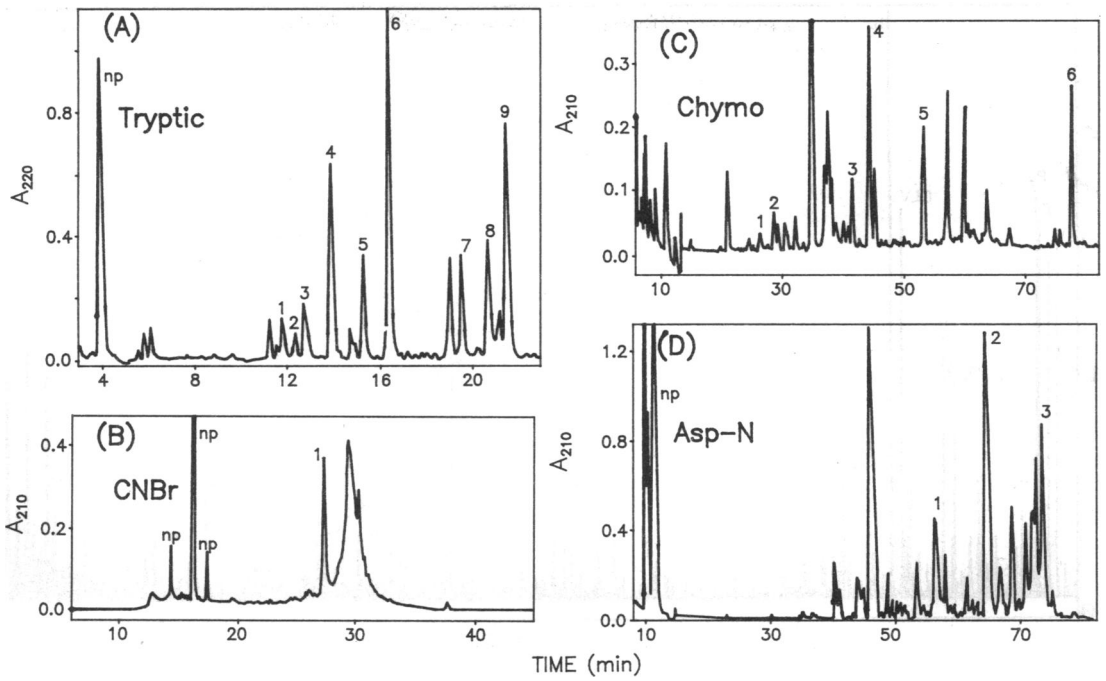

FIG. 2. Reverse-phase HPLC separation of peptide mixtures after partial degradation of bovine GMF-β. (*A*) Tryptic digestion. (*B*) Cyanogen bromide cleavage. (*C*) Chymotryptic digestion. (*D*) Endoproteinase Asp-N digestion. Only the profiles at 210 (or 220) nm are shown, although the original tracings included profiles at 254 nm and 277 nm as well. Only those peaks that were used to construct the final sequence in Fig. 1 are numbered. Peaks that were chemical or HPLC artifacts are marked np (not peptide). Detailed procedures for the cleavage of GMF-β are described below. For tryptic digestion, 50 μg of reduced carboxymethylated GMF-β, dissolved in 500 μl of 1% NH_4HCO_3, was incubated at 37°C for 20 hr with trypsin at a substrate to enzyme weight ratio of 50:1. The digested mixture was reduced to 100 μl on a Speed-Vac and separated by HPLC. For cyanogen bromide cleavage, 4 μg (250 pmol) of reduced S-pyridylethylated GMF-β in 100 μl of 70% (vol/vol) formic acid was mixed with 10 μl of 50 mM cyanogen bromide (>500-fold molar excess with respect to methionine). The mixture was allowed to react overnight in the dark at room temperature under argon. The sample was taken to dryness *in vacuo* and the cleaved peptides were fractionated by HPLC. For chymotryptic digestion, 20 μg of reduced S-pyridylethylated GMF-β was suspended in 100 μl of 1% NH_4HCO_3 and incubated at 37°C with bovine α-chymotrypsin at a substrate to enzyme weight ratio of 50:1. The extent of digestion at 2 hr was monitored by HPLC analysis of a sample of 5% of the reaction mixture. After a 4-hr digestion, the peptide mixture was directly fractionated by HPLC. For endoproteinase Asp-N digestion, 50 μg of reduced carboxymethylated GMF-β was digested with the enzyme in a single 1.5-ml microcentrifuge tube as described by Stone *et al.* (10). All enzyme reagents were of sequencing grade from Boehringer Mannheim.

Table 2. Amino acid composition of terminal peptides of GMF-β

	Amino acid residues, mol/mol of peptide			
	Amino-terminal peptide (Y6)		Carboxyl-terminal peptide (A2)	
Amino acid	Sequence	Amino acid analysis	Sequence	Amino acid analysis
Asx	2	2 (2.2)*	1	1 (1.3)*
Glx	3	3 (3.4)	3	3 (2.9)
Ser	2	2 (2.1)	0	0 (0.0)
Gly	0	0 (0.0)	1	1 (1.0)
His	0	0 (0.0)	1	1 (0.9)
Arg	1	1 (0.9)	1	1 (1.1)
Thr	0	0 (0.0)	1	1 (0.9)
Ala	1	1 (1.0)	0	0 (0.0)
Pro	0	0 (0.0)	0	0 (0.0)
Tyr	0	0 (0.0)	0	0 (0.0)
Val	4	4 (3.6)	0	0 (0.0)
Ile	0	0 (0.0)	0	0 (0.0)
Leu	3	3 (3.0)	3	3 (2.8)
Phe	0	0 (0.0)	2	2 (1.9)
Lys	1	1 (1.1)	1	1 (1.2)
Cys	1	1 (0.8)	0	0 (0.0)
Met	0	0 (0.0)	0	0 (0.0)
Trp	0	ND	1	ND
Total residues, no.	18	18	14†	14

ND, not determined.
*Fractional numbers are in parentheses.
†Not counting tryptophan, for the sake of comparison.

These peptides provided the final connections and completed the linearity from position 1 to position 141.

Establishment of Amino Terminus. Although the composition estimated from the sequence in Fig. 1 is very close to that

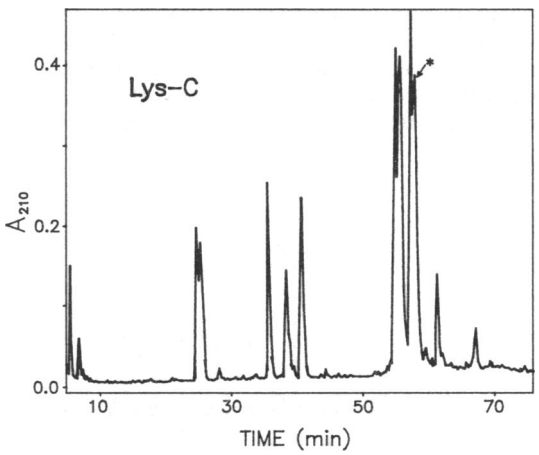

FIG. 3. Reverse-phase HPLC separation of Lys-C digest of bovine GMF-β. Thirty-six micrograms of reduced pyridylethylated GMF-β in 150 μl of 1% NH_4HCO_3 was digested at 37°C for 15 hr with endoproteinase Lys-C (Boehringer Mannheim; sequencing grade) at a substrate to enzyme weight ratio of 50:1 and subsequently fractionated by HPLC. Only the profile at 210 nm is shown, although the original tracing also included profiles at 254 nm and 277 nm. The asterisk indicates the peptide peak selected for mass spectrometry for the determination of the blocked amino-terminal segment.

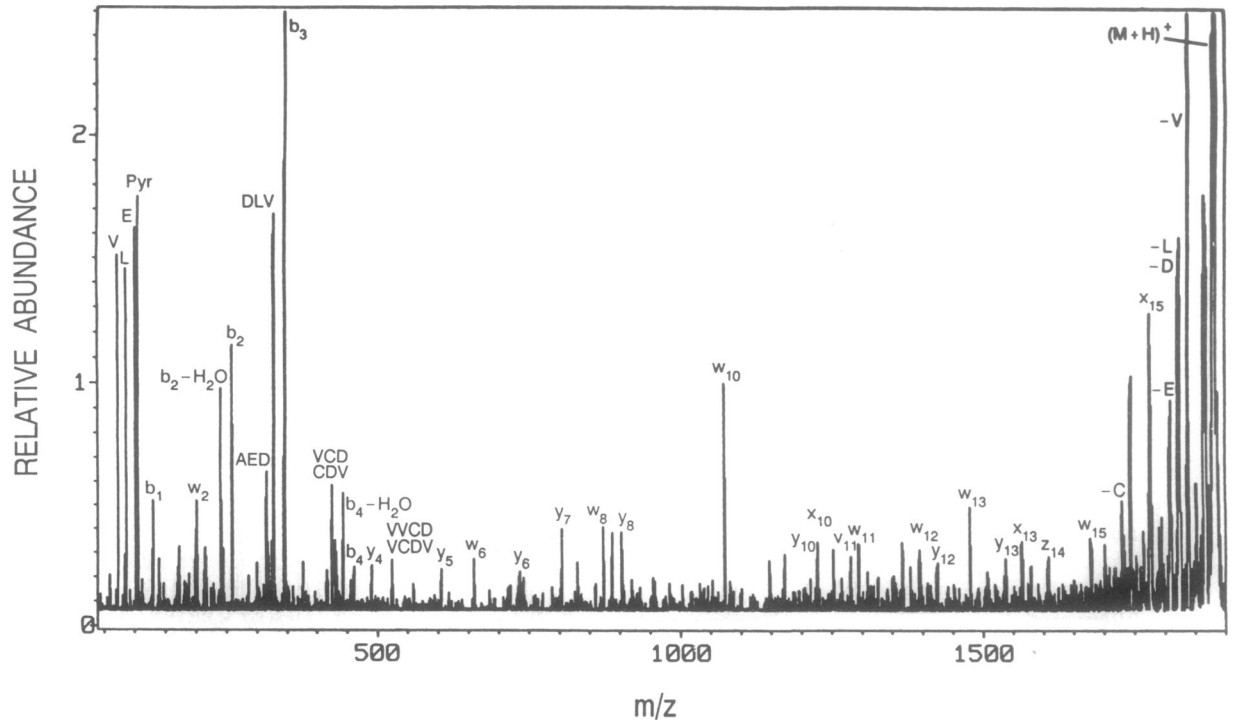

FIG. 4. CID mass spectrum of the protonated peptide molecule [(M + H)$^+$] of the amino-terminal Lys-C peptide of GMF-β (m/z 1881.9). The Lys-C digest of reduced S-pyridylethylated GMF-β was fractionated by HPLC (Fig. 3) and 250 pmol of the peak containing the blocked amino-terminal peptide, predicted to be a 16-residue peptide from Ser-1 to Lys-16 (see Fig. 1), was subjected to tandem mass spectrometry. The FAB mass spectrum (acquired with MS-1) exhibited an abundant (M + H)$^+$ ion at m/z 1881.9 (all ^{12}C-containing isotope, spectrum not shown), consistent with an acetylated hexadecapeptide having a composition of Ala-Asp$_2$-Cys(*S*-pyridylethyl)-Glu$_3$-Leu$_2$-Lys-Ser$_2$-Val$_4$. This protonated molecule was selected with MS-1, collided with He, and the fragment ions thus produced were analyzed by MS-2 resulting in the CID mass spectrum shown. All the major peaks are assignable but not all are labeled for the sake of clarity. Lower-case letters are used to label fragment ions as follows: the b_n ion series corresponds to cleavage of the peptide backbone at the CHRCO-NH bond, with the charge retained on the fragment containing the peptide amino terminus; y_n fragment ions result from cleavage at the same bond but with the charge retained on the fragment containing the peptide carboxyl terminus. The x_n and z_n fragment ion series correspond to cleavage of the peptide backbone at the CHR-CO and NH-CHR bonds, respectively, with the charge retained on the fragment containing the peptide carboxyl terminus. Subscripts are used to locate the amino acid residue position where the cleavage occurred (counting from the carboxyl terminus for x_n, y_n, and z_n ions and from the amino terminus for b_n ions). The w_n and v_n ion series involve partial or complete loss of side chains in addition to peptide backbone cleavage, as explained in refs. 13 and 14. Ions due to internal fragment ions or single amino acid immonium ions are labeled with the standard amino acid single-letter code (upper case). Minus signs in the high-mass region indicate loss of a particular side chain from the protonated peptide molecule [labeled as (M + H)$^+$]. Pyr, pyridylethyl.

obtained by direct analysis of the protein (see Table 1), uncertainty remained regarding the amino terminus because of the block to Edman degradation. That Ser-1 is indeed the amino terminus was finally confirmed by tandem mass spectrometry. To this end, reduced pyridylethylated GMF-β was digested with endoproteinase Lys-C and the resultant peptides were separated by HPLC (Fig. 3). As predicted from Fig. 1, the amino-terminal Lys-C fragment would uniquely contain *S*-pyridylethyl cysteine (absorbance at 254 nm) and lack tyrosine (absence of absorbance at 277 nm). Only one peak met these criteria and it was selected for mass spectrometry after amino acid compositional analysis. (That the major peptide in this peak was amino-terminally blocked was confirmed by its failure to yield a sequence when subjected

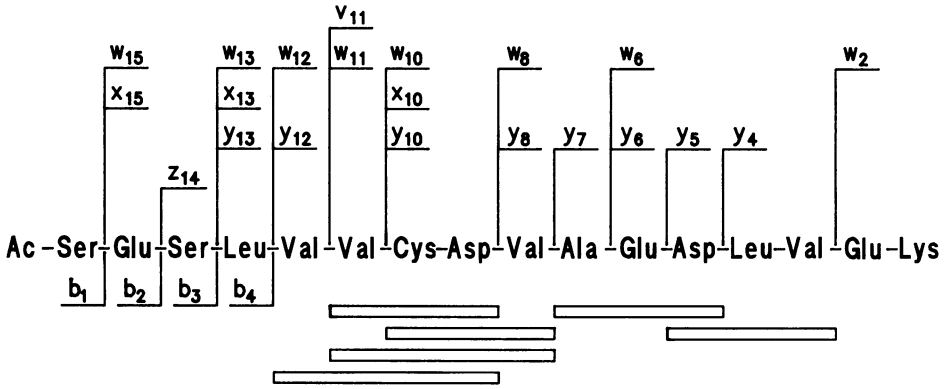

FIG. 5. Sequence construction of the N-blocked hexadecapeptide using data from Fig. 4, showing the observed ion fragments. All labels are explained in legend to Fig. 4. The b_1 fragment ion is at m/z 129.0, consistent with an acetylated serine as the amino-terminal amino acid. Internal fragment ions are represented as rectangular boxes below the peptide chain, matching the location of their sequences. Note that, despite three ambiguous segments (Cys-Asp, Leu-Val, and Glu-Lys), information from the mass spectrum provides strong corroborative evidence to the sequence obtained by Edman degradation (Fig. 1).

to Edman degradation.) Mass spectrometry revealed the following sequence: AcSer-Glu-Ser-Leu-Val-Val-Cys-Asp-Val-Ala-Glu-Asp-Leu-Val-Glu-Lys (Figs. 4 and 5), a hexadecapeptide lying within the sequence of peptide Y6 (Fig. 1). This establishes that an N-acetylated serine was the amino-terminal residue.

Establishment of Carboxyl Terminus. That position 141 (histidine) is the carboxyl terminus was supported by the fact that both T5 and A2 peptides ended with the same histidine residue (Fig. 1). Furthermore, amino acid composition of peptide A2 accounted for every residue detected in the sequence of this peptide fragment, indicating that no additional residue was inadvertently left out (Table 2). Lastly, in a separate experiment, affinity isolation of carboxyl-terminal peptide by immobilized anhydrotrypsin (11) from a tryptic digest confirmed that peptide T5 was the carboxyl-terminal fragment (data not shown).

DISCUSSION

The complete sequencing of bovine GMF-β by Edman degradation presented a problem because of the absence of a free amino terminus. Although we circumvented this problem by fragmenting the protein and sequencing the resultant peptide fragments, the uncertainty of the amino-terminal residue and the blocking agent remained until we resorted to mass spectrometry, which identified that *N*-acetylserine was the amino terminus. We were surprised, however, by the finding that the amino terminus revealed by mass spectrometry was the same as that observed by chymotryptic digestion. We suspect that the N-acetyl group was accidentally removed during incubation with chymotrypsin. But since deacetylation is not a known action of this enzyme, the reason for the unblocking is unexplained.

Comparing GMF-β with other proteins in the Protein Identification Resource data base showed no significant homology, a finding that, to our knowledge, suggests that GMF-β does not belong in any characterized protein superfamily. Nonetheless, this does not preclude the possibility that a family of which GMF-β is a member exists. Future investigation should clarify this point. The molecular size of GMF-β is certainly within the usual range of growth factors (i.e., between 10 and 20 kDa) and is much smaller than the size of most enzymes. The demonstration of specific binding sites on responsive cells (unpublished data) lends support to the argument that GMF-β acts directly on the cells and not indirectly by modifying some other growth factors or extracellular enzymes, as for glial-derived nexin (16).

While this work was in progress, a human cDNA for GMF-β was cloned, sequenced, and expressed in *Escherichia coli* (17). Except for the lack of amino-terminal blocking, the recombinant human GMF-β is identical to bovine GMF-β by all criteria tested, including amino acid sequence, amino and carboxyl termini, and immunologic and biologic activities. The complete conservation of the sequences of GMF-β from the two species implies a strong evolutionary constraint on the structure of this protein and is consistent with the wide distribution of GMF-β among the vertebrates (7).

The discovery of growth factors that regulate the survival, proliferation, and/or maturation of cells brought to light aspects of intercellular communication germane to the understanding of development and regeneration. Since these factors have multiple actions and overlapped target cells, it is necessary to chemically define each of these regulators before their combinatorial and permutative effects can be meaningfully analyzed. The elucidation of the complete sequence of GMF-β provides an absolute identity for the protein and serves as a solid foundation upon which future investigations on its chemical and biological mechanisms can be made.

We thank B. D. Fink, C. A. Russo, and J. L. Delp for technical assistance in the preparation of GMF-β; R. J. Davenport, R. A. Robinson, and D. Andrews for their expert technical assistance in the fragmenting, amino acid analysis, and sequencing of GMF-β; B. V. Plapp and L. S. Tobacman for critical reading of the manuscript; and L. Facto for the preparation of graphs. This work was supported by the following grants to R.L.: Department of Veterans Affairs Merit Review Award, Grant BNS-8917665 from the National Science Foundation, and Grant DK-25295 from the Diabetes-Endocrinology Research Center. Tandem mass spectrometry was carried out by I. A. Papayannopoulos and C. E. Costello at the Massachusetts Institute of Technology Mass Spectrometry Facility, supported by the National Institutes of Health Division of Research Resources (Grant RR0317 to K. Biemann).

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