Purification and Primary Structure of a Polypeptide with Multiplication-stimulating Activity from Rat Liver Cell Cultures

HOMOLOGY WITH HUMAN INSULIN-LIKE GROWTH FACTOR II*

Hans Marquardt and George J. Todaro
From The Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland 21701

Louis E. Henderson and Stephen Orozsan
From The Biological Carcinogenesis Program, Frederick Cancer Research Center, Frederick, Maryland 21701

A low molecular weight polypeptide with multiplication-stimulating activity (MSA) was isolated from serum-free media conditioned by a clone of normal Buffalo rat liver cells. The purification of MSA was achieved by gel permeation chromatography on Bio-Gel P-10 in 1 M acetic acid and followed by reversed phase high pressure liquid chromatography on aBondapak C18 support using a linear gradient of acetonitrile in 0.05% trifluoroacetic acid. The primary structure of MSA has been determined. MSA is a single chain polypeptide of 67 residues, with a calculated molecular weight of 7,484, and displays 93% homology with the functionally related human insulin-like growth factor II (IGF-II). A comparison between the sequences of rat MSA and human IGF-II reveals only five amino acid substitutions. Based on the extensive amino acid sequence homology, we propose the term rat IGF-II for this newly isolated polypeptide.

The normal somatic growth which occurs during the preadolescent period is believed to be mediated in part by a family of insulin-like polypeptides, designated somatomedins (1-3). Several growth hormone-dependent polypeptides with properties of somatomedins have been purified from human plasma, based on their capacity to stimulate sulfate uptake in cartilage or to exert an insulin-like effect on responsive tissues: somatomedin A (4-6) somatomedin C (7, 8), and two distinct insulin-like growth factors (IGF-I and IGF-II)\(^1\) (9, 10). Complete amino acid sequence analyses have been published for IGF-I (11) and IGF-II (12) and partial sequence data for somatomedin C (7).

Multiplication-stimulating activity refers to a group of polypeptides which stimulate DNA synthesis in and multiplication of normal fibroblasts in vitro. The isolation and partial purification of MSA from serum-free medium conditioned by a Buffalo rat liver cell line (BRL-3A) (13) has been reported (14-17), and the functional relationship of MSA to the human somatomedins and insulin-like growth factors has been demonstrated (7, 18, 19). The structural relationship of the somatomedins was suggested by their high degree of immunological cross-reactivity, as demonstrated in radioimmunoassays developed for IGF-I (20), IGF-II (20), somatomedin C (21), and MSA (22). We describe in this report an improved procedure for the purification of a low molecular weight component of MSA, and report the amino acid sequence. The data clearly show an extensive structural homology between this rat MSA and human IGF-II.

The site of biosynthesis of IGF-II is unknown. It has been reported (23) that fetal rat liver explants release MSA-related polypeptides, suggesting that IGF-II may be produced at least in part by the liver. Also cultured human fibrosarcoma cells (24-26), bovine chondrocytes (27), and WI-38 human fibroblasts (28) produce somatomedin-like activity. It is not clear whether IGF-II production is under the control of pituitary growth hormone (20). The findings described here, that a cloned line of rat liver cells produces a close relative of human IGF-II should allow studies on its biosynthesis and how it might be hormonally regulated.

EXPERIMENTAL PROCEDURES\(^2\)

Source of MSA—MSA was purified from serum-free medium conditioned by a line of normal Buffalo rat liver cells, BRL-3A (13), clone BRL-T. The BRL-T clone was obtained from M. M. Rechler and S. P. Nissley, National Institutes of Health, Bethesda. Cells were grown in roller bottles containing Dulbecco's modified Eagle's medium with 10% calf serum. The cells were washed for 1 h with 50 ml of serum-free Waymouth's medium. This and a second collection of supernatant fluid, 24 h later, were discarded. Subsequent collections were made every other day, or every 3rd day, for a 3-week period. The medium was collected by decantation, and clarified by centrifugation at 105,000 \( \times \) g for 30 min at 4 °C. The supernatant, after high speed centrifugation, will be referred to as Buffalo rat liver-conditioned medium.

Dialysis and Centrifugation—Buffalo rat liver-conditioned medium was dialyzed for 60 h against 0.1 M acetic acid in Spectrapor 3 dialysis tubing (3,500 molecular weight cutoff, Spectrum Medical Industries, No. 132720). The supernatant was centrifuged at 105,000 \( \times \) g for 1 h at 4 °C. The pellet was discarded. The supernatant was concentrated by lyophilization and reconstituted in 2.5 ml of 1 M acetic acid/liter of original Buffalo rat liver-conditioned medium.

Chromatography on Bio-Gel P-10—The supernatant containing\(^2\)

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^1\)The abbreviations used are: IGF, insulin-like growth factor; MSA, multiplication-stimulating activity; BRL, Buffalo rat liver; rp HPLC, reversed phase high pressure liquid chromatography; PTH, phenylthiohydantoin.

\(^2\)Portions of this paper (including parts of "Experimental Procedures," parts of "Results," Figs. 5 to 7, and Tables II to V) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80 M-2682, cite author(s), and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

6859
MSA-competing activity was further purified by gel permeation chromatography on a column (2.5 x 85 cm) (420 ml bed volume) of Bio-Gel P-10 (200 to 400 mesh, Bio-Rad). The column was equilibrated with 1 M acetic acid. Samples of protein (60 to 75 mg) in 1 M acetic acid (5 ml) were applied to the column. To ensure a constant flow rate, the column effluent was regulated at 12 ml/h with a peristaltic pump. 3.7-ml fractions were collected. Aliquots were lyophilized for determining MSA-competing activity and growth-promoting activity. Fractions comprising the major MSA-competing activity with an apparent molecular weight of 6,800 were pooled, and concentrated by lyophilization.

Reversed Phase High Pressure Liquid Chromatography—The final purification of MSA was achieved by rp HPLC, as previously published (29). A Waters ALC/GPC model 244 liquid chromatography system was used which included two M-6000 solvent delivery pumps, an M-660 solvent programmer, a U6K sample injector, and a model 450 variable wavelength detector. All separations were performed on a μBondapak C18 reversed phase column (10 μm, 4 mm x 30 cm), also from Waters.

A linear acetonitrile gradient composed of 0.05% trifluoroacetic acid in water as starting buffer and 0.045% trifluoroacetic acid in acetonitrile as limit buffer was used. The column was operated at a flow rate of 1 ml/min at 40°C; the effluent was monitored continuously at 220 nm, and absorbance was recorded on a Beckman 10-in. linear log potentiometric recorder at a chart speed of 0.2 inch/min. The column effluent was collected in 1-ml fractions. Aliquots were lyophilized for MSA competition studies. Pools of fractions comprising the major MSA-competing activity were concentrated by lyophilization.

Radioceptor Assay—Purified MSA was labeled with Na125I by a modification of the chloramine-T method (30), as described (26). The 125I-MSA-binding assays were performed on subconfluent cell cultures of Kirsten-murine sarcoma virus-transformed normal rat kidney cells, as previously described (24).

Results

Purification of MSA—To remove serum proteins, BRL-T cells were extensively washed with Waymouth’s medium prior to their culture in serum-free medium. Culture conditions were such that at the end of the culture period more than 90% of the cells were viable and attached as monolayers.

During the dialysis of Buffalo rat liver-conditioned medium against acetic acid, there was some precipitation of inactive material which was subsequently discarded. The partially purified Buffalo rat liver-conditioned medium was subjected to gel permeation chromatography on Bio-Gel P-10. The column was eluted with 1 M acetic acid to dissociate growth-promoting activity from the binding protein(s). A representative chromatogram is illustrated in Fig. 1. The bulk of the protein (protein elution pattern not shown) was eluted in the exclusion volume of the column. Three peaks of MSA-com-

Fig. 1. Gel permeation chromatography of Buffalo rat liver-conditioned medium on Bio-Gel P-10 (420-ml bed volume). Elution pattern of 75 mg of protein (acid-soluble protein from 2 liters of Buffalo rat liver-conditioned medium). The elution was performed with 1 M acetic acid. One-μl aliquots of the indicated fractions were lyophilized and assayed for MSA-competing activity (C——C) by the radioceptor assay. The following proteins and peptides were used to construct standard plots of log molecular weight versus elution volume: bovine pancreatic ribonuclease A (RNAase A), Mw = 13,700; lima bean trypsin inhibitor (T. Inh.), Mw = 8,400; bovine pancreatic insulin (Insulin), Mw = 5,700. The elution volumes of the standard peptides are indicated.

Fig. 2. High pressure liquid chromatography of MSA. Chromatography on a μBondapak C18 column in 0.05% trifluoroacetic acid in water. Elution pattern of 190 μg of peptide from P-10-C (Fig. 1). Elution was achieved with 0.045% trifluoroacetic acid in acetonitrile. The solid line gives the peptide absorbance at 220 nm. The dashed line denotes the acetonitrile concentration.

RESULTS

Assay for Growth-promoting Activity—MSA activity was estimated by determining the incorporation of [3H]thymidine into triploid, subconfluent cell cultures of serum-deprived, subconfluent normal rat kidney cells, clone 49F (31), as described (26).

Fig. 3. Amino acid sequence of rat MSA and a schematic outline of the data supporting the sequence. Principle tryptic peptides in MSA are indicated by T. Sequence assignments for residues based on semi automated Edman degradation are indicated by an arrow (→). Sequence assignments for residues were also provided by carboxypeptidase Y (―) and carboxypeptidase B (―) digestion. Tentative assignments are indicated by brackets. Data supporting the sequence are given in the miniprint section.
peting activity were usually observed. The largest activity peak (P-10-A) was capable of directly binding 125I-labeled MSA; those fractions thus appear to contain a binding protein for MSA. Fractions with MSA-competing and growth-stimulating activity (P-10-B and P-10-C) were well separated from each other and had apparent molecular weights of 8,500 and 6,800, respectively, as determined by gel permeation chromatography on a calibrated Bio-Gel P-10 column in 1 M acetic acid. The peaks of growth-promoting activity paralleled the peaks of MSA-competing activity very closely. Fractions with an apparent molecular weight of 6,800 (P-10-C) were pooled, lyophilized, and further purified by rp HPLC.

Pool P-10-C was reconstituted in 0.05% trifluoroacetic acid in water, and then chromatographed on a µBondapak C₈ column. A typical elution pattern is illustrated in Fig. 2. The MSA-competing and growth-stimulating activity (data not shown) co-eluted with the major absorbance peak at 34% acetonitrile. Fractions, indicated with a bar, were pooled, lyophilized, and further analyzed. The pattern shown in Fig. 2 reveals that, in the solvent system used, some minor contaminating UV-absorbing material was effectively separated from the main peak of MSA-competing activity. The yield of purified MSA was 165 µg/liter of Buffalo rat liver-conditioned medium.

Amino Acid Composition of MSA—The amino acid composition of MSA is shown in Table I and compared with that of human IGF-II (12). The closest approximation to integral values for all residues was obtained by assigning a total of 67 residues (M, = 7,484). The composition of MSA showed a striking similarity with the amino acid composition of IGF-II, isolated from human plasma, revealing only two amino acid residue differences: human IGF-II contains one more valine and one less aspartic acid.

Amino Acid Sequence of MSA—The amino acid sequence of MSA is shown in Fig. 3. The structure was determined by microsequence analysis, as described in detail in the miniprint section. Briefly, the amino acid sequence of the first 44 amino acid residues was determined by semiautomated Edman degradation (32) of 22 nmol of unmodified MSA. The PTH-derivatives were identified by rp HPLC (33). The remainder of the amino acid sequence was deduced from microsequence analysis data obtained from 4 to 6.5 nmol input of tryptic peptides isolated from a digest of 9 nmol of 5-[14C]N-amidino-MSA and from the results of carboxypeptidase digestion of selected tryptic peptides and unmodified MSA. Tryptic peptides were isolated by rp HPLC using volatile solvents (29).

**DISCUSSION**

In this report, a procedure for the purification of an MSA from the rat liver cell clone, BRL-T, has been described. For its isolation from serum-free conditioned medium, methods were selected to maintain the biological activity. Purification was by gel permeation chromatography and rp HPLC in volatile solvents. Lyophilized aliquots of column fractions were soluble in binding buffer, and were analyzed for growth-promoting and MSA-competing activity.

On gel permeation chromatography of Buffalo rat liver-conditioned medium in 1 M acetic acid, the binding protein-containing peak (P-10-A) was well separated from the two major growth-stimulating peaks (P-10-B and P-10-C) with apparent molecular weights of 8,500 and 6,800, respectively, which co-eluted with two peaks of MSA-competing activity. Acid conditions were required for this purification step of MSA from Buffalo rat liver-conditioned medium to dissociate the complex of binding protein and growth-promoting polypeptides. Further purification of P-10-C by rp HPLC resulted in highly purified MSA. No contaminating peptide material was found by Edman degradation of this preparation.

The cell clone, BRL-T, from a line of normal Buffalo rat liver cells, BRL-3A, produced a variety of MSA-competing peptides of different molecular weights. An M, = 16,300 MSA polypeptide has previously been identified (17) that could possibly be a precursor of the small molecular weight peptides produced by cultured Buffalo rat liver cells. Despite the existence of multiple molecular weight species, the low molecular weight MSA-competing activity represented the major growth-stimulating activity of Buffalo rat liver-conditioned medium. The heterogeneity of pool P-10-B observed in our laboratory and by others (17) could possibly reflect incomplete enzyme-catalyzed hydrolysis of a pre-prohormone polypeptide after synthesis. The apparent molecular weight of MSA (pool P-10-C) was 6,800, as determined by gel permeation chromatography on a Bio-Gel P-10 column in 1 M acetic acid, calibrated with peptides of known molecular weights. The estimated molecular weight of MSA is in agreement with the amino acid composition if calculated from the sum of the residue weights of the polypeptide chain. MSA is a peptide of 67 residues with a molecular weight of 7,484. A comparison of the amino acid compositions of MSA with human IGF-II indicates a high degree of similarity. Both components lack histidine and methionine. Despite minor differences in the contents of aspartic acid, and valine, no major differences were seen. The absence of methionine in MSA and IGF-II distinguished these peptides from IGF-I (12). In contrast to results presented here, previously reported amino acid compositions of MSA (16, 17) failed to show a relationship to IGFs.

The amino acid sequence of MSA, as presented in Fig. 3, was determined by semiautomated Edman degradation of the native polypeptide, and by Edman degradation and carboxypeptidase digestion of selected tryptic peptides. Undetected residues (Cys-9, Cys-21, Thr-62) or tentatively assigned resi-
polypeptide.

---

**REFERENCES**

Structure of a Rat Multiplication-stimulating Activity

Experimental Procedures

Protein Determination - Total protein was determined (35) using bovine serum albumin as a standard. Prior to protein determination, the starting material was analyzed against phenylthiohydantoin standards. The amino acid analysis was performed using the amino acid analyzer equipped with a P/ACE computing integrator. Cysteine was determined as cysteic acid after performic acid oxidation at 24%. For 0%, or as carbamylated-cysteine followed by acid hydrolysis. The amino acid analysis data were analyzed by the National Institutes of Health Data System-5 computer using a program similar to that described by Bayer et al. (36).

Sequence Determination - For reduction, 0.1 ml of 6 M Tris-HCl buffer (pH 9.5) containing 0.03 M dithiothreitol (DTT) and 0.05 M EDTA (pH 7.5) was added, and the resulting solution was stirred at 24°C for 4 h under nitrogen. For the subsequent elution, 0.1 M acetic acid of 0.05 M lodoacetamide-2-mercaptoethanol, CFA, 200 uL of specific activity, 0.1 M Tris-HCl (pH 7.5) and 0.05 M sodium bicarbonate buffer (pH 9.5) containing 0.03 M dithiothreitol (DTT) were added, and the solution was stirred for 2 h. The carboxamidomethylated MSA was assayed on a 0.5 x 20 cm column of Bio-Gel P-2 (200-400 mesh), equilibrated with 0.1 M acetic acid at 24°C and pH 7.4. The specific activity of C-carboxamidomethylated MSA was 2.94 mg/mmol of cysteine.

Acidolation - To acetylate tryptic peptides to give acetyllysines, the Cys-containing groups of the tryptic peptides in MSA were modified as described in the Methods section. A representative chromatogram in Fig. 6. The sequence of the amino-terminal 44 residues of MSA was established on the basis of the automated degradation of 22 mmole of native peptide. The yields of the PTH-amino acids identified from this degradation are shown in Fig. 5. The amino acid sequence deduced from these data is presented in Fig. 3. At each step in the degradation, PTH-amino acids were seen from the last residue in the sequence. This observation indicated that approximately 10% of the total yield of PTH-amino-terminal sequence, and 49% had amino-terminal cysteine residues were not identified in this experiment. The results presented in Table 3 were tentatively identified as serine or threonine.

Tryptic Fragments of MSA

Nine mg of C-carboxamidomethylated MSA were digested with TPCK-trypsin. The mixture of the tryptic peptides was separated by RP-HPLC on a O.Bondapak C18 column, as described in "Experimental Procedures." A representative chromatogram is shown in Fig. 6. The major absorbance peaks, containing Cys-containing peptides, were pooled, lyophilized, and then for RP-HPLC analysis using more severe gradient conditions. The chromatogram resulting from the rechromatography of peaks 8, 9, and 10 is shown in Fig. 7A, 7B, and 7C, respectively. Peptides containing cysteine were pooled as indicated. Aliquots were taken for amino acid analysis, acidolysis, and digestion. The cysteine residues were identified by the amino acid sequences. The major absorbance peaks were identified as tryptic peptides.
The residue composition was determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.

The amino acid compositions were determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.

The amino acid compositions were determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.

The amino acid compositions were determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.

The amino acid compositions were determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.

The amino acid compositions were determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.

The amino acid compositions were determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.

The amino acid compositions were determined on samples hydrolyzed in 6 M HCl at 110°C for 24 h. The number in brackets are those found in the sequence.