PURIFICATION OF THE PROTEIN THAT INDUCES CELL DIFFERENTIATION TO MACROPHAGES AND GRANULOCYTES

Moshe GUEZ and Leo SACHS
Department of Genetics, Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

Three different proteins have been isolated that effect the development of hematopoietic cells. Erythropoietin [1–4] effects erythrocytes, thymosin [5–7] effects lymphocytes and the protein that we now call MGI [8] induces from single undifferentiated hematopoietic cells the formation of colonies with mature macrophages and granulocytes [8–15]. Erythropoietin [3–4] and thymosin [7] have been purified and we have previously reported [8] the 600-fold purification of MGI from serum-free conditioned medium from a tissue culture line of mouse cells. It was shown that MGI is a protein, some properties of the protein were described and it was found that purification resulted in loss of biological activity. Activity was regained by addition of a low molecular weight cofactor that was also present in conditioned medium, and the cofactor could be substituted by adenine or adenine-containing nucleotides. The cofactor, adenine or adenine-containing nucleotides showed no activity without MGI [8,9].

The present experiments describe the purification to homogeneity in SDS-polyacrylamide gel electrophoresis, of the protein MGI from conditioned medium from the line of cultured mouse cells, its molecular weight, amino acid composition and labeling by acetylation. The purified protein gave a single band on SDS-polyacrylamide gel electrophoresis with a mobility equivalent to a molecular weight of 68 000. After adding adenine or the cofactor from conditioned medium, it induced the formation of both macrophage and granulocyte colonies, with an inducing activity of 0.5 ng per colony. The protein had no detectable cysteine, cystine, hexosamines or sugars and it has been labeled by acetylation without loss of biological activity.

2. Materials and methods

2.1. *Bioassay*

Induction of colony formation was assayed on cells cloned in 0.33% agar medium on a 0.5% agar medium base in 50 mm Petri dishes [10–12]. Samples of different volumes from the solutions to be tested were made up with Eagle's medium with a 4-fold concentration of amino acids and vitamins (EM) to a constant volume of 5.5 ml. To this was added an equal volume of a mixture containing agar, 2 × EM and useless otherwise stated, inactivated (56°C for 30 min) horse serum, to give a final concentration of 0.5% agar and 10% horse serum. The cells for cloning were generally taken from embryo livers of the ICR or SWR strain of mice at about the 17th–19th day of gestation. Cell suspensions were made as described [12] and aliquots of 1.7 ml of the soft agar mixture (0.33%) containing 6×10^4 embryo liver cells were seeded on the 0.5% agar base. Colonies were counted microscopically between 7–10 days after seeding. Each point in an experiment was based on the average from 2–4 plates. The total number of colonies was calculated from the linear portion of the curve obtained after plotting the total number of colonies against MGI concentration [8]. All bioassays, after Diaflo ultrafiltration and subsequent steps in purification, were carried out with addition of 1.25 ml per Petri dish of the cofactor in conditioned medium that is in the outside solution after Diaflo XM-50 followed by Diaflo UM-05 ultrafiltration.

2.2. *Conditioned medium, Diaflo ultrafiltration, column chromatography and gel filtration*

Serum-free conditioned medium from a cloned tissue culture line of mouse cells [8] was harvested after 3–4 days, centrifuged at 10 000 *g* for 15 min to remove debris, and then passed through a 0.45 μm Millipore filter. This solution was concentrated by lyophilization, suspended in distilled water, centrifuged to separate the insoluble matter and the supernatant filtered for concentration with Diaflo membrane XM-50. Chromatography on hydroxyapatite (Bio. Gel HTP from Bio-Rad Labs. Richmond, Calif.) was on columns (1.8 × 10 cm) in equilibrium with 10^{-3} M K-Na phosphate buffer (pH 6.8) eluted by the following stepwise increases in buffer concentration: 10^{-3}, 10^{-2}, 2×10^{-2}, 8×10^{-1}, 5×10^{-1} and 1 M. Chromatography on DEAE-cellulose, (Diethyl-Amino-Ethyl from Bio-Rad, Labs. Calif.) was on columns (0.85 × 10 cm) in equilibrium with 10^{-3}M K-Na phosphate buffer (pH 6.8) eluted by the following stepwise increase in the concentration of phosphate buffer, 10^{-3}, 10^{-2}, 2×10^{-2}, 6×10^{-2}, 10^{-1}, 2×10^{-1} and 5×10^{-1} M. The fractions from hydroxyapatite and DEAE-cellulose were concentrated and treated as described [8]. For gel filtration on Sephadex G-150 (Pharmacia, Uppsala), columns (1 ×90 cm) were equilibrated in phosphate-buffered saline in water (1:10) pH 7.2, at a pressure head of 30 cm. The void volume was determined using Dextran blue 2000. Columns were run at room temperature at a constant head of 30 cm with a flow rate of 12–15 ml per hour. Every fraction after the void volume was assayed for inducing activity.

2.3. *Amino acid analysis, hexosamine and sugar determination*

Protein was determined according to Lowry et al. [16]. Amino acid, glucosamine and galactosamine analysis were made according to Spackman et al. [17] in a Beckman amino acid analyzer. Protein samples were hydrolyzed at 100°C in 6 N HCl. Sugar determinations were made by gas liquid chromatography according to Sawardeker et al. [18] and Derek and Moss [19] and by thin-layer chromatography according to Lato et al. [20, 21].

2.4. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was carried out by the method of David [22]. Six-cm long glass tubes with an inner diameter of 0.5 cm were used with 7.5% gel. Electrophoresis was run at 2 mA per tube for 90 min at 4°C. The gels were stained with 0.5% Amido black solution in 7% acetic acid and destained with the same solvent. To quantitate the radioactive labeled bands, the gel was frozen, cut into 1.0–1.2 mm slices and each slice dissolved in 0.2 ml of 30% H_2O_2 [23], incubated for 4 hr at 60°C. After cooling at room temperature, 0.8 ml of water was added and the sample counted with Triton X-100 toulene scintillation fluid. Stained and unstained gels were run in parallel. Each slice of unstained gel was extracted by grinding with glass powder in 0.5 ml of water, filtered with a 0.45 μm Millipore filter and assayed for biological activity.

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS) was carried out with a single layer of 10% acrylamide according to Weber and Osborn [24] and with two layers (spacer gel) with 7.5% acrylamide in the lower gel according to Laemmli [25]. Bovine serum albumin was obtained from Armour Pharmaceutical Co., ovalbumin and phosphorylase A from Worthington Biochemical Corp. and pyruvate kinase from Sigma Chemical Co.

2.4. *Acetylation of MGI with [^{3}H] acetic anhydride*

MGI was labeled with [^{3}H]acetic anhydride in benzene by the method of Miller and Great [26]. The biologically active peak obtained from a DEAE-cellulose column was lyophilized and labeled with [^{3}H] acetic anhydride in benzene solution with a specific activity of 500 mCi/mmole (Radio-chemicals, Amersham) by keeping for 7 days at 4°C. It was then lyophilized to remove the benzene, resuspended in water and the labeled protein filtered through Diaflo membrane PM-10 to remove the free acetic anhydride.

3. Results

3.1. *Steps in the purification of MGI*

MGI was purified from the serum-free conditioned medium by the following steps: lyophilization, ultrafiltration with a Diaflo XM-50 membrane, chromatography on hydroxyapatite and DEAE-cellulose and then gel filtration on Sephadex G-150 (table 1). All bioassays after Diaflo ultrafiltration, were carried

Table 1
Stages in the purification of MGI.

Solution	mg Protein per ml of	No. of colonies per mg protein	Recovery (%)		Degree of purification	ng Protein per colony
			Biological activity	Protein		
Serum free CM *	0.2	1×10^3	100	100	1	1000
Lyophilized CM	0.3	4.9×10^3	100	23	4.9	140
Diaflo (XM-50) ultrafiltered CM	3.9	1.5×10^4	90–100	10	15	80
Hydroxyapatite peak, 0.08 M	1.6	4×10^4	50–70	0.5	40	20
DEAE-cellulose peak, 0.01 M	0.3	0.6×10^6	30–50	0.15	600	1.5
Sephadex G-50 peak	0.1	1.8×10^6	15–20	0.5	1800	0.55

* CM = conditioned medium containing MGI.

out with addition of 1.25 ml per Petri dish of the cofactor in conditioned medium. As in previous experiments [8], the main peaks of biological activity from the hydroxyapatite and DEAE-cellulose columns were at 0.08 M and 0.1 M phosphate buffer, respectively. There was also a sharp peak of biological activity after the last step of gel filtration on Sephadex G-150. The peak obtained after Sephadex filtration gave a single band on polyacrylamide gel electrophoresis without or with SDS (fig. 1), was 1800-fold purified and had an inducing activity of 0.5 ng protein per colony (table 1). Bioassay of the purified protein with different concentrations of the cofactor from conditioned medium of the addition of adenine have shown (fig. 2) that the concentration of cofactor used, 1.25 ml per Petri dish, to calculate the colony-inducing activity of purified MGI, was at the optimum

Fig. 1. Polyacrylamide gel electrophoresis with SDS. Purified MGI (right), bovine serum albumin (left).

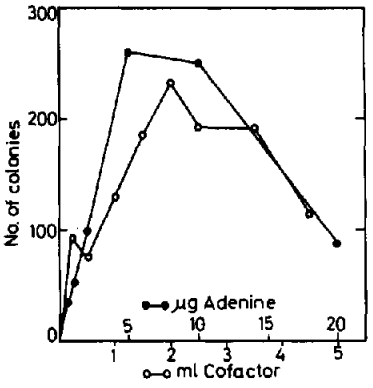

Fig. 2. Induction of colony formation by purified MGI in the presence of (o-o-o) cofactor from conditioned medium (bioassay with horse serum); or (●-●-●) adenine (bioassay with calf serum).

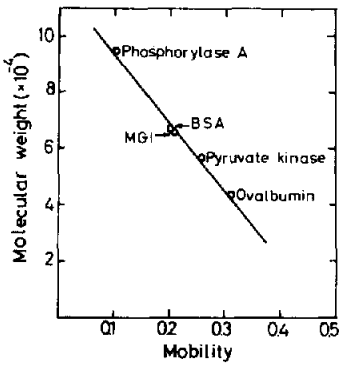

Fig. 3. Molecular weight determination of purified MGI by polyacrylamide gel electrophoresis with SDS, expressed as relative mobility against log. mol. wt.

concentration of cofactor. The cofactor or adenine without MGI showed no activity.

The purified protein induced the development of both macrophage and granulocyte colonies in bioassays with bone marrow cells from adult mice. Three–five μg purified MGI with 1.25 ml cofactor or 2.5 μg adenine per Petri dish, induced the formation of 10% and 20% granulocyte colonies, respectively.

3.2. *Molecular weight and amino acid composition*

The mobility of purified MGI in a 10% acrylamide gel with SDS was similar to that of bovine serum albumin (fig. 3). This indicates that MGI has a molecular weight equivalent to 68 000. Similar results were obtained with 7.5% acrylamide containing SDS. The results of amino acid analysis of purified MGI (table 2) have indicated, that there was no detectable cystine, cysteine, methionine or arginine. The thiobarbitoric assay for sialic acid [27] was negative. There were no detectable hexosamines in the amino acid analyser and no detectable sugars by gas–liquid chromatography and thin layer chromatography.

Table 2
Amino acid composition of purified MGI.

Amino acid	Amino acid residue (%)
Lysine	4.85
Histidine	1.71
Arginine	–
Aspartic acid	10.74
Threonine	7.32
Serine	9.47
Glutamic acid	12.66
Proline	5.10
Glycine	8.39
Alanine	9.74
Cystine (half)	–
Valine	8.09
Methionine	–
Isoleucine	3.70
Leucine	8.04
Tyrosine	3.72
Phenylalanine	6.41

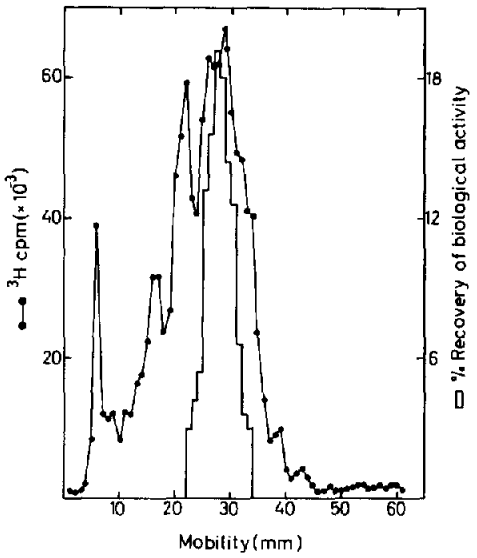

Fig. 4. Polyacrylamide gel electrophoresis of labeled MGI from the active peak from DEAE-cellulose, (•-•-•) ^{3}H cpm; histogram, % recovery of activity.

3.3. *Labeling of MGI*

The biological active peak from DEAE-cellulose column was labeled with [^{3}H]acetic anhydride as described in Materials and methods. The labeled protein had the same colony-inducing activity as the unlabeled protein. As with the unlabeled protein, the labeled MGI gave a sharp peak of biological activity after gel filtration on Sephadex G-150 and on polyacrylamide gel electrophoresis (fig. 4). Twenty-five–fifty μg of purified labeled MGI after the final step of Sephadex filtration, was incubated with 0.1 mg/ml pronase (Calbiochem. A grade) at 37°C for 24 hr in phosphate-buffered saline, pH 7.2, and then filtered

through a Diaflo PM-10 membrane. About 90% of the biological activity was retained after this pronase treatment and filtration.

4. Conclusions

The present results have shown that the purified protein MGI from serum-free conditioned medium from a cloned line of mouse cells, gave a single band on polyacrylamide gel electrophoresis with SDS, with a mobility equivalent to a mol. wt. of 68 000. Erythropoietin and thymosin have been reported to have mol. wt. of 46 000 [4] and 12 600 [7], respectively. Erythropoietin cannot substitute for MGI [28]. The amino acid composition of MGI indicates that this protein does not have cysteine or cystine. There were also no detectable hexosamines or sugars and the biological activity was not destroyed after treatment with pronase. The yield of purified protein was about 0.5 mg from 10 liters of conditioned medium, and the purified MGI had an inducing activity of 0.5 ng per colony. As in previous experiments [8,9], purification of the protein resulted in loss of biological activity and the activity was regained by addition of a low molecular weight cofactor that was also present in conditioned medium or by the addition of adenine.

The purified MGI with the cofactor or adenine induced the formation of both macrophage and granulocyte colonies. It will be of interest to compare the purified MGI from the mouse with the MGI from human sources [29–31], to further characterize the part of this protein that carries the biological activity and how this differs from thymosin and erythropoietin. MGI was labeled by acetylation without loss of biological activity. The availability of purified labeled protein now makes it possible to determine the location of MGI-binding sites on the target and other cells and its mode of action.

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