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Purification and subunit structure of hepatocyte growth factor from rat platelets

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A hepatocyte growth factor (HGF) that stimulates DNA synthesis of adult rat hepatocytes in primary culture was purified as a homogeneous material from platelets of 1000 rats by a four-step procedure: stimulation of its release from platelets by thrombin, cation-exchanger fast protein liquid chromatography (FPLC) on a Mono S column, heparin-Sepharose CL-6B chromatography, and reverse-phase HPLC on a C_4 column. The purified HGF stimulated DNA synthesis of adult rat hepatocytes in primary culture at 1 ng/ml and was maximally effective at 5 ng/ml, being about twice as potent as EGF at this concentration. HGF did not stimulate DNA synthesis of Swiss 3T3 cells. It was found to be a heat- and acid-labile protein that was inactivated by reduction with dithiothreitol. The purified HGF had a molecular mass of 82 kDa, as estimated by SDS-PAGE, and was found to be a heterodimer which dissociated into a large subunit of 69 kDa and a small one of 34 kDa by SDS-PAGE under reducing conditions. These biological and chemical properties showed that HGF was not identical with any known growth factors, including platelet-derived growth factor (PDGF).

Hepatocyte growth factor; Primary cultured hepatocyte; Platelet; Heterodimer

1. INTRODUCTION

Liver is known to regenerate actively after partial hepatectomy and there have been many attempts to identify a humoral factor that stimulates proliferation of liver cells (hepatotropic factor) [1]. However, no such factor has yet been purified or characterized [2], because no simple, reproducible and sensitive in vitro method is available for its assay. In the last decade, several studies including our own [3–8] have demonstrated that adult rat hepatocytes in primary culture, which retain many liver-specific functions and respond to various hormones in the same way as in vivo [9–11], can proliferate at low cell density when insulin and epidermal growth factor (EGF) are added to the culture medium. Thus, primary cultures of adult rat hepatocytes are a suitable in vitro assay system for identification and purification of a hepatotropic factor. Using this in vitro assay system, we previously purified a hepatotropic factor that strongly stimulated DNA synthesis of mature rat hepatocytes from the serum of partially hepatectomized rats, and named this factor hepatocyte growth factor (HGF) or hepatotropin [12]. Subsequently, several groups have found that HGF is present at a high concentration in rat platelets and is released on aggregation of the platelets [12–14]. Recently, we purified HGF from rat platelets and demonstrated that it is a new growth factor differing from platelet-derived

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growth factor (PDGF) in some chemical and biological properties [15]. However, we could not determine the subunit structure of this HGF from rat platelets, because our preparation was not completely homogeneous.

Here, we report on the complete purification of HGF from rat platelets with reverse-phase HPLC on a C_4 column as the final purification step. We also report studies showing that HGF is a heterodimer with a molecular mass of 82 kDa on SDS-PAGE and is composed of two polypeptides of 69 and 34 kDa, respectively.

2. MATERIALS AND METHODS

2.1. *Materials*

A mono S column and heparin-Sepharose CL-6B were obtained from Pharmacia (Uppsala). DE 52 was purchased from Sigma. [*methyl*-^{3}H]Thymidine (54.2 Ci/mmol) was from the Radiochemical Centre. Mouse EGF was purified from the submaxillary glands of male adult mice by the method of Savage and Cohen [16]. Recombinant human EGF was a gift from Earth Chemical Co., Akoh.

2.2. *Primary culture of adult rat hepatocytes*

Parenchymal hepatocytes were isolated from adult Wistar rats (180–250 g) by perfusion of the liver in situ with collgenase. The isolated cells were cultured as monolayers in Williams medium E supplemented with 5% calf serum, 2 nM insulin and 1 μM dexamethasone [17]. The initial cell density was 2.5×10^5 cells/2 cm diameter wells of Linbro multi-well plastic dishes coated with type I collagen. After 4 h, the medium was changed to serum-free Williams medium E with aprotinin (bovine pancreatic trypsin inhibitor) at 0.1 μg/ml.

2.3. *Assay of HGF activity*

The test sample or insulin (0.1 μM) and EGF (20 ng/ml) were added to the culture 20 h after plating. After further incubation for 12 h, [^{3}H]thymidine (2.5 μCi/ml, 0.27 Ci/mmol) was added to the medium and culture was continued for 24 h. Incorporation of [^{3}H]thymidine into DNA was determined as in [4]. One unit of HGF activity is defined as that equivalent to half the amount of EGF required for maximum stimulation of DNA synthesis of adult rat hepatocytes in primary culture.

2.4. *Secretion of HGF from rat platelets*

Rat platelets were isolated as 99% purity in a yield of about 10^{10} platelets from the blood of 100 rats as described [15]. The platelets were suspended at 1×10^{10} platelets/ml in phosphate-buffered saline (PBS) and incubated with 2 U/ml of pure thrombin for 10 min at 25°C to induce aggregation. After 10 min, phenylmethylsulfonyl fluoride was added at a final concentration of 1 mM. Aggregates of platelets were precipitated by centrifugation and the resulting supernatant was used as the starting material for purification of HGF.

2.5. *Flow-through on DE 52 column*

The material released from platelets of 300–400 rats was applied to a DE 52 column (1.6 × 14 cm) previously equilibrated with 10 mM Hepes buffer (pH 7.2) containing 0.15 M NaCl and 2 mM $CaCl_2$. The flow-through fraction was collected and adjusted to pH 8.5 with 1 M Tris.

2.6. *Mono S FPLC*

The flow-through fraction from the DE 52 column was applied to a Mono S column (1 × 10 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.5) containing 0.15 M NaCl, 10 mM Hepes, and 2 mM $CaCl_2$. The column was washed with the same buffer and then HGF was eluted with a linear gradient of 0.15–1.0 M NaCl in the same buffer at a flow rate of 60 ml/h. For assay of HGF activity, 20-μl aliquots of all fractions were diluted with 24 vols of a solution of bovine serum albumin (BSA, 2.5 mg/ml) in PBS and sterilized by filtration through a 0.22 μm filter (Millex GV, Millipore).

2.7. *Affinity chromatography on a heparin-Sepharose CL-6B column*

Active fractions obtained from the Mono S FPLC step were pooled, adjusted to pH 7.5 with 1 N HCl, and diluted with 3 vols distilled water. The diluted sample was applied to a heparin-Sepharose CL-6B column (1 × 5 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl. The column was washed with 10 column volumes of the equilibration buffer, and then HGF was eluted with 30 ml of a gra-

dient of 0.3–2.0 M NaCl at a flow rate of 20 ml/h. For assay of HGF activity, 10 μl aliquots of all fractions were diluted with 49 vols of a solution of 2.5 mg/ml BSA in PBS and sterilized by Millipore filtration as described above.

2.8. *Reverse-phase HPLC on a C_4 column*

Active fractions obtained by heparin affinity chromatography were combined and applied to a Hi-pore Rp 304 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. HGF was eluted with a linear gradient of 0–90% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fractions of 1 ml were collected and promptly neutralized by adding 0.2 ml of 0.1% sodium bicarbonate. HGF activity was assayed after 200-fold dilution of the samples with BSA and sterilization by Millipore filtration.

2.9. *SDS-polyacrylamide gel electrophoresis*

SDS-PAGE of purified HGF was performed in 12.5% acrylamide gel as described by Laemmli [18]. After electrophoresis, the gel was fixed and stained with silver [19].

3. RESULTS

3.1. *Purification of HGF from rat platelets*

Previously we purified HGF from rat platelets via a three-step procedure [15]; namely, we stimulated its release from platelets by thrombin, and then purified it by cation-exchanger FPLC on a Mono S column and heparin-Sepharose CL-6B chromatography. However, this purified HGF was still contaminated with other proteins as judged by SDS-PAGE. Therefore, in this work we tried to purify HGF to homogeneity by reverse-phase HPLC. For this the HGF preparation obtained from the platelets of 1000 adult rats by our previous three-step procedure was applied to a Hi-pore Rp 304 column (4.6 × 250 mm, Bio-Rad C_4 column) equilibrated with 0.1% trifluoroacetic acid. Fig.1 shows the elution pattern of HGF. A large peak of contaminating protein with no activity was eluted with 40% acetonitrile, and then on increasing the acetonitrile concentration to 43%, HGF was eluted as a sharp peak. All fractions of eluate were neutralized with 1% sodium bicarbonate just after elution from the Hi-pore Rp 304

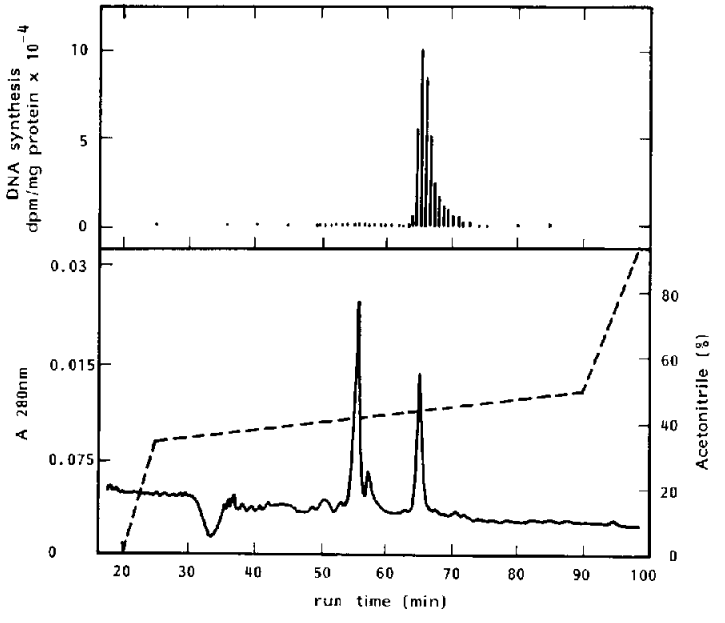

Fig.1. Reverse-phase HPLC of HGF. A sample of 5 ml of the active fraction (5 ml) from a heparin-Sepharose column was injected into a Hi-pore Rp 304 column and eluted with a gradient 0–90% acetonitrile in 0.1% trifluoroacetic acid. Fractions of 1 ml were collected, promptly neutralized with 0.1% $NaHCO_3$ and their activity measured. (– – –) Relative A_{280}; (——) acetonitrile gradient; (bars) HGF activity.

column, since HGF is an acid-labile protein. When the fractions were promptly neutralized, we could obtain HGF in high yield even by reverse-phase HPLC under acidic conditions with 0.1% trifluoroacetic acid. Results of a typical purification are summarized in table 1. About 23 μg pure HGF was obtained from platelets of 1000 rats. The specific activity of the final preparation was calculated to be 46.1 $\times$ 10^4 U/mg protein. Thus, this preparation had much higher specific activity than that of our previous preparation (38.5 $\times$ 10^4 U/mg protein). The overall recovery of HGF activity was 24% with 15897-fold increase in specific activity over that in the crude preparation released from platelets.

Fig.2 shows the dose-response curve to the purified HGF obtained by reverse-phase HPLC. HGF had a detectable effect at a concentration as low as 1 ng/ml and was maximally effective at 5 ng/ml. The maximum activity was almost twice as potent as that of EGF. Its effect was additive or synergistic with those of insulin and EGF, as reported previously. Purified HGF did not stimulate DNA synthesis in Balb/c or Swiss 3T3 cells. HGF activity was almost completely inactivated by heating (100°C, 3 min), or treatment with 1 N acetic acid or trypsin, as reported in [15].

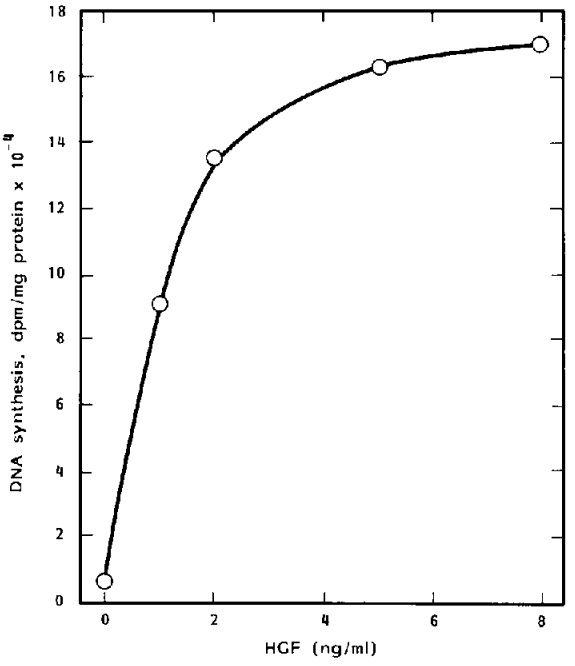

Fig.2. Dose-response curve for effect of pure HGF on DNA synthesis of adult rat hepatocytes in primary culture. Pure HGF, obtained by reverse-phase HPLC, was diluted with BSA (2.5 mg/ml) and sterilized. Then its HGF activity at the indicated concentrations was assayed. Protein was measured by amino acid analysis. Values are means for triplicate dishes.

3.2. *Analysis of the subunit composition of HGF*

The HGF obtained by reverse-phase HPLC was subjected to SDS-PAGE. HGF migrated as a single band in a position corresponding to a molecular mass of 82 kDa, as shown in fig.3. The HGF activity, assayed as activity for stimulating DNA synthesis of adult rat hepatocytes in primary culture, in eluates of gel slices was also detected in the position of the 82 kDa band on SDS-PAGE under non-reducing conditions (not shown).

Table 1

Purification of HGF from platelets of 1000 rats

Purification step	Protein (mg)	Activity (U $\times$ 10)	Spec. act. (U/mg protein $\times$ 10)	Purity (-fold)	Recovery (%)
Supernatant from platelets	1541	44.3	0.029	1	100
DE 52 run-through	1482	42.3	0.028	1	95
Mono S FPLC	5.93	28.8	4.86	168	65
Heparin-Sepharose	0.057	13.8	241	8310	31
Reverse-phase HPLC	0.023	10.6	461	15897	24

Material released from platelets of 1000 rats was used as starting material. HGF activity was measured at four different concentrations and was determined from the linear portion of the dose-response curve. Protein concentration was calculated from the absorbance at 280 nm up to the step of Mono S FPLC and by amino acid analysis in subsequent steps

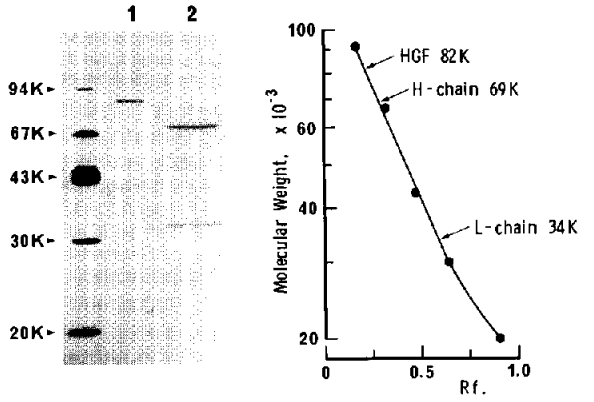

Fig.3. SDS-PAGE of purified HGF under reducing and non-reducing conditions. HGF samples (20 μl, 0.1 μg) with or without treatment with 2-mercaptoethanol were subjected to SDS-PAGE as described in section 2. The molecular mass (in kDa) markers used were as follows: glycogen phosphorylase, 94; BSA, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20.1; lactalbumin, 14.4. Lane 1, unreduced HGF; lane 2, reduced HGF.

However, on SDS-PAGE under reducing conditions, purified HGF gave two bands with molecular masses of 69 and 34 kDa, respectively (fig.3). Neither fraction of reduced HGF had any biological activity. These findings indicate that HGF consists of two different polypeptide chains, linked covalently by a disulfide bond to form a molecule with a heterodimer. The value of 82 kDa calculated as the molecular mass of HGF from its mobility on SDS-PAGE under non-reducing conditions, may be apparent, because calculated values become less accurate with increase in molecular mass. Thus the value of 103 kDa obtained as the sum of the molecular masses of two subunits may be more accurate.

4. DISCUSSION

Previously, we demonstrated that the serum of rats after partial hepatectomy contained a hepatotropic factor that stimulated DNA synthesis of mature hepatocytes in primary culture, and we named this factor hepatocyte growth factor (HGF) or hepatotropin [12]. We found that the HGF activity in rat serum increased time-dependently after partial hepatectomy, reaching a maximum of about 5-fold the initial level 24 h after the operation. Moreover, we recently observed a marked increase in HGF activity in rat plasma after liver injury by administration of D-galactosamine or CCl_4 to rats (in preparation). The serum-derived HGF was a heat- and acid-labile protein with a molecular mass of over 100–150 kDa, as judged by gel filtration on Sephadex G-200. Later, it became evident that HGF is stored in rat platelets and released on their aggregation induced by thrombin treatment [12–14]. Recently, using purified HGF from rat platelets we found that the serum- and platelet-derived HGFs have almost the same properties. Moreover, Selden et al. [20] observed that HGF activity increased markedly in the serum of patients after partial hepatectomy for removal of hepatomas and Nakayama et al. [21] observed high HGF activity in sera of patients with fulminant hepatic failure. Furthermore, Gohda et al. [22] partially purified HGF from the plasma of patients with fulminant hepatic failure, and found that human HGF has very similar biological and chemical properties to rat HGF purified from platelets. These findings indicate that HGF is the hepatotropic factor that has long been suspected to exist.

We have reported that HGF purified from rat platelets stimulated DNA synthesis of adult rat hepatocytes in primary culture but not of fibroblasts such as Balb/c or Swiss 3T3 cells [12]. In contrast, various known growth factors, such as fibroblast growth factor, insulin-like growth factors I and II, transferrin, and thrombin as well as PDGF, did not stimulate DNA synthesis in cultured rat hepatocytes, but stimulated the growth of 3T3 cells. Moreover, here we found that the molecular mass and subunit structure of HGF differ from those of various other growth factors. HGF has a high molecular mass and is a heterodimer composed of a large subunit of 69 kDa and a small subunit of 34 kDa. To our knowledge, HGF is the largest growth factor that has been isolated so far. In addition to PDGF, platelets contain several mitogens such as platelet basic protein [23], connective tissue activating peptide III [24], and β-type transforming growth factor [25]. All these growth factors are heat- and acid-stable factors and are mitogenic to cells derived from mesenchymal tissues. Moreover, the β-type transforming growth factor inhibits DNA synthesis in primary cultured hepatocytes

stimulated by insulin and EGF [26–28]. Thus, HGF also differs from various other platelet-derived growth factors besides PDGF in cell specificity and chemical properties including molecular size. Therefore, these findings indicate that HGF is a new growth factor for adult rat hepatocytes in rat platelets. Furthermore, it seems to be a main factor in the stimulation of liver regeneration after partial hepatectomy and liver injury.

HGF may be useful clinically for enhancing liver regeneration after surgical operations on the liver and for treatment of patients with hepatitis. For this purpose, a method is required for large-scale preparation of human HGF. Molecular cloning of cDNA coding for human HGF and preparation of an expression vector are necessary for elucidating the chemical structure of HGF and for large-scale preparation of human HGF for clinical use. We are now cloning HGF cDNA from a cDNA library constructed from poly(A) RNA of rat megakaryocytes using a DNA probe corresponding to the N-terminal part of the amino acid sequence of rat HGF.

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