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Hepatocyte growth factor-like protein is identical to macrophage stimulating protein

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Although the hepatocyte growth factor-like protein (HLP) shares a 50% homology with the hepatocyte growth factor, the biological function of HLP has remained unknown. Addition of conditioned medium of COS-7 cells transfected with the expression plasmid for HLP cDNA to cultures of resident peritoneal macrophages induced specific activation of macrophages, and the factor which stimulates macrophages was purified from the conditioned medium. The purified protein showed M_r of 85 kDa on SDS-PAGE, and this M_r is in agreement with that of macrophage-stimulating protein (MSP) previously purified from human serum, as well as with the predicted M_r of HLP. Amino acid composition of the purified protein coincided with the compositions of human HLP and MSP. Together with the finding that the partial amino acid sequences of MSP are highly homologous to that of HLP, we conclude that the biological function of HLP is to activate macrophages and that HLP and MSP are identical molecules.

Hepatocyte growth factor; Hepatocyte growth factor-like protein; Macrophage stimulating protein; HGF-family

1. INTRODUCTION

Hepatocyte growth factor (HGF) was first discovered and isolated as a potent mitogen for adult rat hepatocytes in primary culture [1]. Purification [2–4] and molecular cloning [5–9] of HGF revealed that HGF is a disulfide-linked heterodimer molecule composed of a 69 kDa α-subunit containing four kringle domains and a 34 kDa β-subunit containing a serine protease-like domain, thereby possessing 38% homology in amino acid sequence with plasminogen. HGF is now known to be a mesenchymal-derived pleiotropic factor (reviewed in [10,11]) which acts as a mitogen [9,12], motogen (enhancement of cell motility) [13] and morphogen [14] predominantly for various epithelial cells, and it also functions as a tumor suppresser [15–17]. HGF is also known to function as 'trophic factor' for regeneration of various organs (reviewed in [18]), including the liver [19,20], kidney [21–23], and lung [24]. On the other hand, a high affinity cellular receptor for HGF was identified to the c-*met* proto-oncogene product with an intracellular tyrosine kinase domain [25–27].

While many growth factors were classified into certain families, according to structural similarity, growth factors belonging to the HGF family have only recently been identified. cDNA encoding a unique protein with a domain structure similar to that of HGF was isolated from a human liver cDNA library, and the putative protein was named hepatocyte growth factor-like protein (HLP) [28]. HLP has four kringle domains and a serine protease-like domain and their amino acid sequences have a 50% homology with HGF [28]. While researching the biological activity of HLP, we observed that partial amino acid sequences of macrophage stimulating protein (MSP), previously purified from human serum [29], were highly homologous with those of HLP [28].

Based on the amino acid sequence homology, we asked whether HLP and MSP may be identical molecules. We now report convincing evidence that HLP and MSP are indeed identical. The biological function of HLP is to induce activation of macrophages.

2. MATERIALS AND METHODS

2.1. *cDNA cloning of human HLP and construction of the expression plasmid*

Human HLP cDNA was cloned by polymerase chain reaction (PCR). Single-strand cDNAs were synthesized from 1 μg of human liver poly(A) RNAs, using random primers in the presence of M-MLV reverse transcriptase (BRL, Gaithersburg, MD). The upstream half 1.1 kb and downstream half 1.2 kb of human HLP cDNA were amplified from single-strand cDNAs by PCR using primers as follows: Sense primer-1 (human cDNA nucleotide sequence −57 to −39), 5′-TCGAAGCTTCACAACTTCCCGGATGG-3′; antisense primer-1 (1077–1094), 5′-ACGTCGTCTGTACAACGC-3′; sense primer-2 (932–949), 5′-TCCCTCATCAGCACCGAT-3′; antisense primer-2 (2146–2163), 5′-CTGTCTAGACCAAGGCATATGGCATCA-3′ [28]. PCR products were sequenced, then the upstream half and the downstream half were ligated to construct a full-size open reading frame of HLP cDNA at the *Bbe*I site present in both fragments [28].

Mammalian expression plasmid, pUC-SRα was constructed by inserting an *Sal*I fragment of pcDL-SRα [30] which contains the SRα promoter and poly(A) signal into the pUC19 plasmid. Human HLP

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cDNA expression plasmid, pUC-SRα/HLP was constructed by inserting a 2.2 kb full-size open reading frame of human HLP cDNA downstream of SRα promoter.

2.2. *Cell culture and transfection of plasmid*

COS-7 cells were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS). Ten μg of plasmid was mixed with 400 μg/ml of DEAE-Dextran and transfected into COS-7 cells in DME medium containing 100 μM chloroquine. Following a culture period of 48 h, the medium was changed to serum-free DME medium, the cells were further cultured for 72 h, and the medium was used for purification of HLP.

2.3. *Northern blot analysis*

Total RNA was prepared from COS-7 cells by the acid guanidium phenol chloroform method [31] at 24 h after the transfection of plasmid. Five μg of RNAs were denatured and electrophoresed in 1% agarose/formaldehyde gel. RNAs were transferred to a nylon membrane (Amersham) and hybridized with the ^{32}P-labeled 2.2 kb human HLP cDNA fragment.

2.4. *Detection of MSP activity*

MSP activity was measured based on the ability to induce attachment and spreading of macrophages isolated from C3H mouse [32]. Resident peritoneal macrophages suspended in RPMI-1640 medium supplemented with 10% FCS at 5×10^5 cells/ml were added to each well of a 96-well plate, and test samples were added to the culture. After culturing the cells for 6 h, the adhesion and spreading of macrophages were observed. One unit of MSP activity was defined as the reciprocal of the maximal dilution fold of samples within a concentration range in which MSP activity was obvious after the dilution.

2.5. *Purification of HLP/MSP from conditioned medium*

Serum-free conditioned medium (360 ml) obtained from COS-7 cells transfected with pUC-SRα/HLP was dialyzed against 10 mM phosphate buffer (pH 6.7) containing 0.2 M NaCl, and loaded onto 48 ml of heparin Sepharose (Pharmacia, Uppsala) column equilibrated with the same buffer at 4°C. The column was washed with 200 ml of the same buffer containing 0.01% Tween 80, and bound proteins were eluted with a 240 ml linear gradient of 0.2–1.2 M NaCl in the same buffer. MSP active fractions obtained from affinity chromatography were diluted with 4 volumes of 10 mM phosphate buffer (pH 6.7) and loaded onto a 4 ml CM Sepharose (Pharmacia) column equilibrated with the same buffer containing 0.1 M NaCl. The column was washed with 50 ml of the buffer, after which the bound proteins were eluted with a 80 ml linear gradient of 0.1–0.3 M NaCl in 10 mM phosphate buffer (pH 6.7). After active fractions from CM Sepharose chromatography (13 ml) were concentrated using Centriprep (Amicon, Inc., Beverly, MA) to 0.5 ml, 0.25 ml of concentrated sample was loaded onto a Superose-12 column (Pharmacia) equilibrated with 10 mM phosphate buffer (pH 6.7) containing 0.1 M NaCl and fractionated at 0.5 ml/tube.

2.6. *SDS-PAGE and amino acid analysis*

SDS-PAGE was performed by the method of Laemmli [33] using a 10–20% gradient gel. Proteins were visualized by silver staining, using a Wako silver staining kit (Wako Pure Chemical, Osaka). MSP active fractions pooled from FPLC using a Superose-12 column were further purified by reverse-phase high-performance liquid chromatography (HPLC), using a Phenyl-5PW column (Tosoh, Tokyo). Ten μg of purified protein was hydrolyzed with HCl and analyzed using an automated amino acid analyzer (HITACHI L-8500).

3. RESULTS

3.1. *MSP activity in conditioned medium of COS-7 cells*

The expression plasmid of HLP (pUC-SRα/HLP) or mock plasmid (pUC-SRα) was transfected into COS-7 cells and the expression of human HLP mRNA was analyzed by Northern hybridization (Fig. 1). A transcript of about 2.5 kb of HLP mRNA was specifically expressed in COS-7 cells transfected with pUC-SRα/HLP, but it was not detected in COS-7 cells transfected with mock plasmid, pUC-SRα. Thus, HLP mRNA was specifically expressed in COS-7 cells transfected with the expression plasmid containing HLP cDNA.

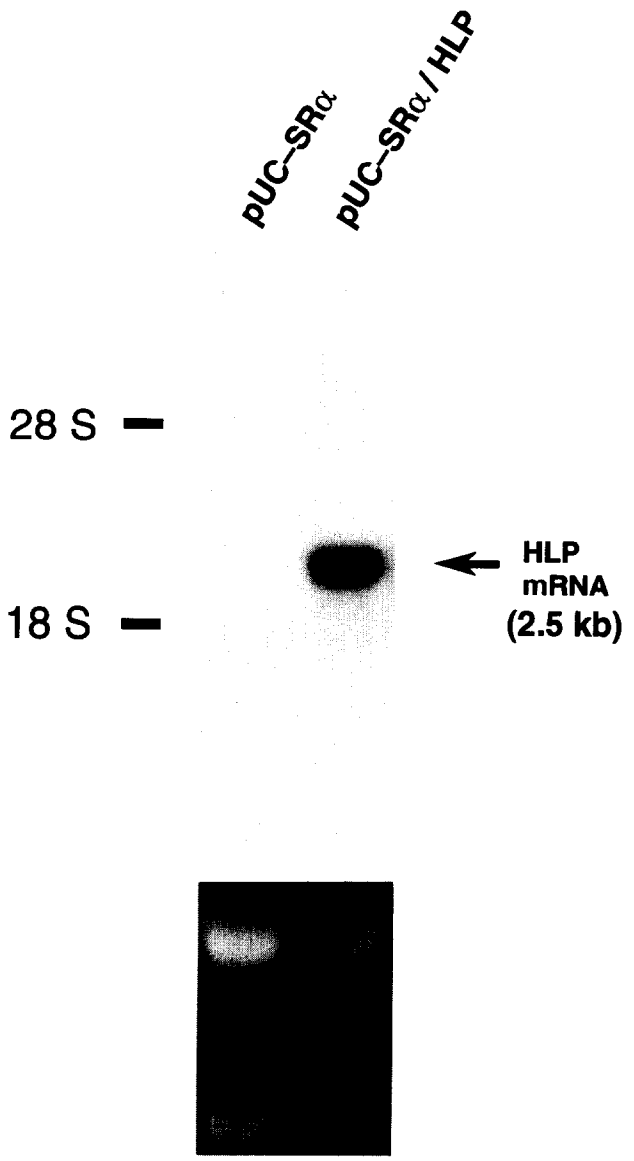

Fig. 1. Expression of human HLP mRNA in COS-7 cells transfected with the expression plasmid encoding human HLP cDNA. The expression plasmid of HLP (pUC-SRα/HLP) or mock plasmid (pUC-SRα) was transfected into COS-7 cells and the expression of human HLP mRNA was analyzed by Northern hybridization. Total RNA was prepared from the cells at 24 h after transfection. Ribosomal RNAs stained with ethydium bromide are shown in the lower photograph, to indicate the amount of RNAs loaded on to the gel.

To examine whether COS-7 cells expressing HLP mRNA produce a factor possessing MSP activity, the

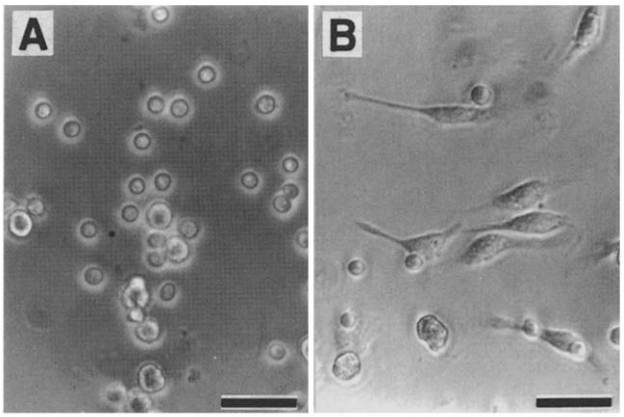

Fig. 2. Activation of mouse peritoneal macrophages by the addition of conditioned medium of COS-7 cells. The conditioned medium of COS-7 cells transfected with pUC-SRα (A) or pUC-SRα/HLP (B) were added to culture of macrophages and cells were further cultured for 6 h. Bars represent 50 μm.

conditioned medium of COS-7 cells was assayed for MSP activity. When conditioned medium from COS-7 cells expressing HLP mRNA was added to cultures of murine peritoneal macrophages, the cells rapidly adhered and spread out, within 6 h (Fig. 2). In contrast, the addition of conditioned medium from COS-7 cells transfected with mock plasmid induced no morphological changes in the macrophages. Thus, the culture medium-secreted HLP from COS-7 cells contains high levels of MSP activity.

3.2. *Purification of a factor with MSP activity from the conditioned medium*

To characterize and purify the factor with MSP activity present in the conditioned medium, the conditioned medium of COS-7 cells transfected with pUC-SRα/HLP was first applied to a heparin Sepharose column. More than 90% of the proteins applied to the column were not adsorbed and MSP activity was not detected in the flow-through fraction. When proteins were eluted with an increasing concentration of NaCl, MSP activity was detected in fractions eluted with 0.4–0.6 M NaCl (Fig. 3A). MSP-active fractions from the heparin Sepharose column werre subsequently applied to a CM Sepharose column. When adsorbed proteins were eluted with the NaCl concentration gradient, MSP activity was eluted with about 0.15 M NaCl, overlapping with a major peak at A_{280} (Fig. 3B). MSP-active fractions from cation-exchange chromatography were further purified by molecular sieve chromatography, using a Superose-12 column. After this chromatography, MSP activity overlapped with a single peak of A_{210} (Fig. 3C).

Table I summarizes the purification of a factor possessing MSP activity. Starting from 360 ml of conditioned medium, the factor was purified 800-fold with a 9% recovery and 16 μg of purified protein was obtained.

Table I

Summary of purification of the factor with MSP activity

Purification step	Protein (μg)	Activity ($U \times 10^{-3}$)	Specific activity (U/μg)	Recovery (%)	Purity (fold)
Conditioned medium	1.49×10^5	36	0.24	100	1
Heparin-Sepharose	2.05×10^3	18	8.78	50	36.6
CM-Sepharose	45.2	5.6	124	15.6	517
Superose 12	16.2	3.2	198	8.9	825

Protein concentration was determined from the absorbance at 280 nm of each fraction. One unit of MSP activity was defined as the reciprocal of the maximal dilution fold of samples within a concentration range in which MSP activity was evident after the dilution.

3.3. *SDS-PAGE and amino acid analysis*

Profiles of proteins at each step of purification were analyzed by SDS-PAGE under non-reducing condition (Fig. 4). Purified protein with MSP activity showed a single band with an apparent M_r of 85 kDa. When SDS-PAGE was performed under reducing conditions, HLP shows a single band of 85 kDa (not shown), thereby indicating that HLP was purified as a single-chain proform. This value estimated with SDS-PAGE is in good accord with 80,325 Da, the M_r calculated from the nucleotide sequence of human HLP cDNA [28], and close to 70 kDa, the M_r of purified MSP from human serum [29]. Since there are three potential N-linked glycosylation sites in the amino acid sequence of human HLP [28], differences among these M_r may be, at least, due to differences in the degree of glycosylation.

For further confirmation, the amino acid composition of the purified protein was analyzed and compared with the composition of human HLP deduced from the nucleotide sequence [28], as well as that of MSP purified from human serum [29] (Table II). The amino acid composition of the purified protein totally coincided with the composition of HLP deduced from the nucleotide sequence indicating that the purified protein with MSP activity is human HLP. Although significant differences are obvious in a few amino acids between the purified protein (HLP) and MSP, these differences may be caused by impurity in MSP preparation. Except for a few amino acids, amino acid compositions of both proteins are very much the same, suggesting that HLP and MSP are identical molecules.

4. DISCUSSION

The present paper provides evidence that HLP has potent biological acitivity to activate macrophages and that HLP is identical to MSP, as based on: (i) the medium from COS-7 cells expressing human HLP mRNA specifically contained a factor which activates macrophages; (ii) the M_r of a protein purified from the condi-

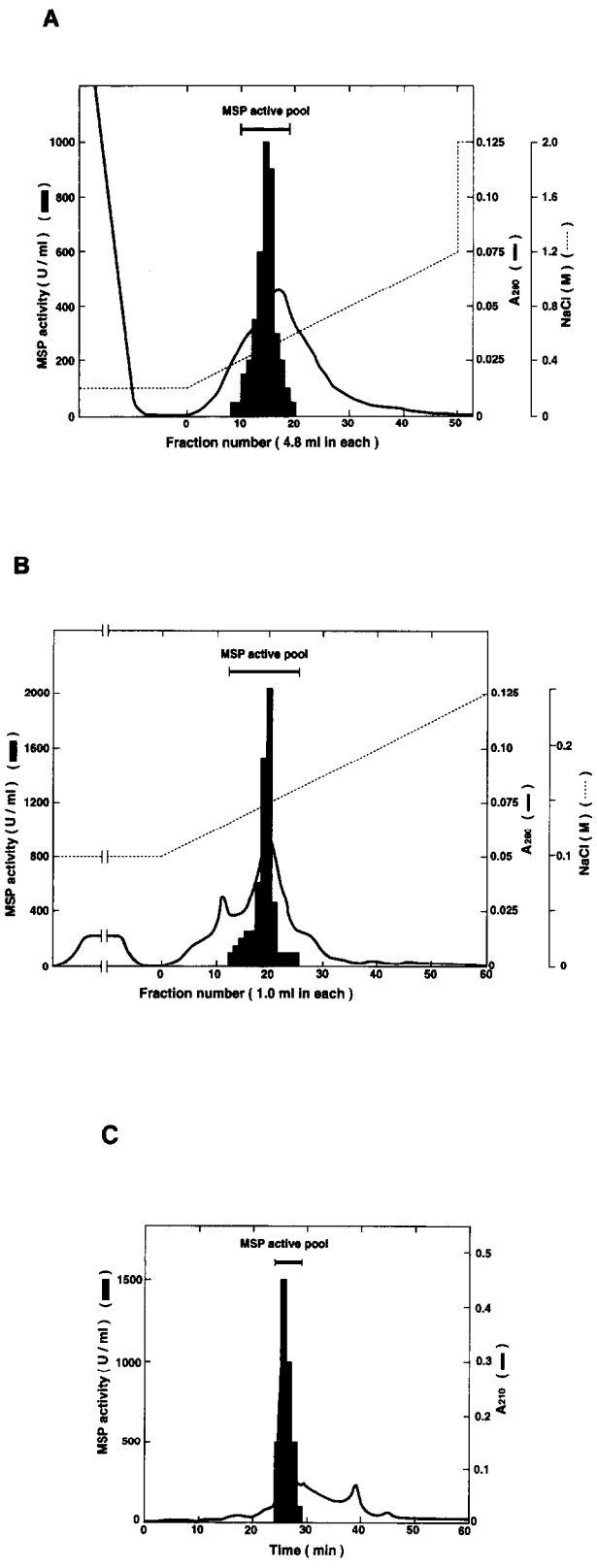

Fig. 3. Elution profiles of MSP activity from conditioned medium of COS-7 cells transfected with pUC-SRα/HLP on column chromatographies. (A) Affinity chromatography with heparin Sepharose; (B) Cation-exchange chromatography with CM Sepharose; (C) Molecular sieve chromatography with Superose-12. Fractions indicated by bars were pooled for the next purification step.

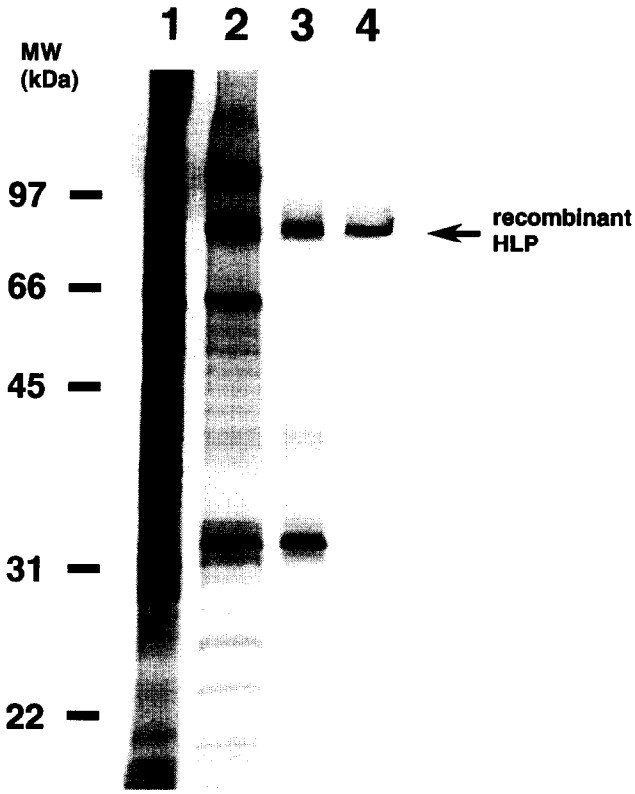

Fig. 4. Protein profiles of MSP-active fractions of each purification step after SDS-PAGE. Lane 1, conditioned medium of COS-7 cells; lane 2, active pool after heparin Sepharose; lane 3, active pool after CM Sepharose; lane 4, active pool after Superose-12. After electrophoresis, proteins were fixed, and visualized by silver staining. Standard proteins used for the determination of M_r were phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (22 kDa).

tioned medium, based on the biological activity to stimulate macrophages, corresponded to 85 kDa, a value with close similarity with the predicted M_r for human HLP, as well as with the M_r of MSP; (iii) the amino acid composition of the 85 kDa protein significantly corresponded with compositions of human HLP and MSP.

Although HLP has the same domain structure as HGF, several proteins that contain kringle domains and a serine protease-like domain have been characterized as proteolytic enzymes involved in blood coagulation and fibrinolysis, including factor XII, prothrombin, tissue plasminogen activator, urokinase plasminogen activator, and plasminogen [34]. Although both HGF and HLP also have a serine protease-like domain, three triad amino acids (His, Asp, Ser) found in the active site of a serine protease domain are substituted by other amino acids, in both HGF and HLP [5,28]. Consistent with these substitutions, HGF has no proteolytic activity [5], and thus it is unlikely that HLP has proteolytic activity. Therefore, HGF and HLP are considered to belong to a distinct cytokine family, because these molecules are functionally distinguishable from proteolytic enzymes, although both have a significant structural similarity to proteases.

Processing of single-chain HGF to two-chain heterodimeric HGF is known to be coupled with the activation of pro-HGF and serum-derived serine protease is responsible for the activation [35]. Similarly, purified single-chain HLP did not show MSP activity when assayed under serum-free conditions (not shown). Since MSP activity was usually assayed under serum-containing conditions, single-chain pro-HLP might be activated during the assay for MSP. Indeed, when single-chain HLP was pretreated with serum, MSP activity was induced in a dose- and time-dependent manner, even when assayed under serum-free conditions (not shown). Single chain pro-HLP may be activated through processing by serum-derived protease(s).

Many growth factors are classified into distinct families based on structural similarity, and growth factors that belong to the same family exert biological activities through either the same receptor or through structurally highly related receptors. Because the HGF receptor is a c-*met* proto-oncogene product [25–27], HLP may exert activity through a receptor structurally related to the c-*met*/HGF receptor. In this context, a novel receptor-type tyrosine kinase 'RON' which belongs to the c-*met*/HGF receptor family has been cloned but the natural ligand is unknown [36]. It is worth noting that the gene for RON is located at locus p21 on human chromosome 3 [36], and this locus corresponds to that of the human gene for HLP [28]. Taken together with the finding that the genes for HGF (7q21) and c-*met* (7q21–31) are both located on the long arm of human chromosome 7 [37–40], the ligand for RON may possibly be HLP.

HGF is a mesenchymal-derived factor with pleiotropic action. It induces mitogenesis [9,12] and motogenesis [13,41,42], and has unique morphoregulatory functions, including the induction of tubule formation in vitro [14,43,44]. This factor has also been well characterized as a 'trophic factor' for regeneration of various organs such as liver [18,19], kidney [21–23], and lung [24]. Thus, HGF is a uniquely suited molecule to construct the normal tissue architecture during development and tissue regeneration. While biological and physiological functions of HLP are the subject of extensive research, we speculate that HLP may be involved in self-defense responses during inflammatory reactions caused by infections and tissue injury, by activating macrophages. Finally, we propose that known and yet to be identified members of the HGF family may play important roles in unique self-defense systems responsible for the regeneration of tissues and organs, and for the maintenance of tissue homeostasis.

Table II

Amino acid composition of 85 kDa protein

	Mol %		
Amino acid	Purified protein	HLP[1]	MSP[2]
Asp	9.46	9.88	6.98
Thr	6.12	6.64	6.36
Ser	4.35	4.94	5.12
Glu	12.8	12.4	10.1
Pro	8.74	8.64	9.92
Gly	12.5	9.88	18.1
Ala	6.11	5.40	9.15
Val	7.27	6.79	8.06
Met	0.91	1.54	1.24
Ile	1.76	2.31	2.02
Leu	7.63	8.18	7.29
Tyr	2.45	2.93	1.71
Phe	3.45	3.86	2.79
His	3.61	3.86	2.64
Lys	3.73	3.40	3.41
Arg	9.16	9.26	5.12

[1]Deduced from the nucleotide sequence without cysteine and tryptophane.
[2]Reported by Skeel et al. [29].

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