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Internalization and degradation of hepatocyte growth factor in hepatocytes with down-regulation of the receptor/c-*Met*

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Hepatocyte growth factor (HGF) promotes proliferation of cultured hepatocytes by its interaction with cell surface receptors. In this paper, we examined the metabolic fate of HGF using hepatocytes. Kinetic analysis using [^{125}I]HGF showed that 40% of surface-bound HGF was rapidly internalized in hepatocytes within 30 min at 37°C. Under these conditions, the amount of HGF-bound c-*Met*, the high-affinity receptor, decreased from the cell surface. Furthermore, the internalized HGF was degraded and released from the cells. These results indicate that cell surface-bound HGF is internalized and degraded by the receptors, including c-*Met*, on hepatocytes.

Hepatocyte growth factor; Scatter factor; c-*Met*; Internalization

1. INTRODUCTION

Hepatocyte growth factor (HGF), also known as scatter factor, is a heterodimer polypeptide held together by a disulfide bond [1–3]. The heavy chain (54–65 kDa) contains four kringle structures and the light chain (31.5–34.5 kDa) contains a serine protease-like domain. The HGF sequence is similar to that of plasminogen [4–7]. HGF has potent mitogenic activity for a variety of cells including cultured hepatocytes [1,8,9]. Furthermore, HGF has other biological activities such as dissociation of epithelial cells [10], growth-inhibition of some tumor cell lines [11] and tubule formation by Madin-Darby canine kidney epithelial cells [12].

High- and low-affinity HGF receptors have been identified in various cell lines and in cultured hepatocytes [13–15]. The high-affinity receptor is the c-*met* proto-oncogene product. The c-*Met* protein possesses a tyrosine kinase domain that is autophosphorylated by HGF [13,15,16], suggesting that the intracellular signaling for HGF is transmitted from the high-affinity receptor. The low-affinity receptor for HGF is considered to be a heparin-like molecule such as heparan sulfate proteoglycan (HSPG), which is commonly found at the cell-surface and the extracellular matrix [13–15,17].

Receptor-mediated internalization is the means of transporting ligands, including growth factors, into the cytoplasm of the target cells. It is essential for degradation of ligands and recycling of the receptors [18–21]. In vivo analysis, HGF is rapidly bound in rat liver, and degraded [22], suggesting that it is internalized in the target cells. In this study, we examined the internalization and metabolic fate of HGF in hepatocytes in vitro. The results showed that HGF bound to the receptors is rapidly internalized, degraded and finally released from the cells.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Reagents were obtained as follows: protein-A Sepharose CL-4B from Pharmacia LKB Biotechnology Inc.; bis(sulfosuccinimidyl)-suberate (BS^3) from Pierce Chemical Co.; gelatin from Bio-Rad; phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, soybean trypsin inhibitor, insulin, dexamethasone, aprotinin, heparin (average molecular mass: 4,000–6,000 Da), gentamicin, Williams' medium E from Sigma; Dulbecco's modified Eagle's medium (DMEM) from Gibco. An antiserum against the murine c-*Met* protein (anti-*Met* antiserum) [15] was a gift from Dr. M. Komada and Dr. N. Kitamura (Institute for Liver Research, Kansai Medical University, Japan). Radioiodinated recombinant human HGF was prepared as described previously [23,24].

2.2. *Primary culture of adult rat hepatocytes*

Parenchymal hepatocytes were isolated from adult male Wistar rats, weighing about 200 g, by the method of Seglen [24]. The cells were plated in collagen-coated 6-well plastic dishes or collagen-coated plastic dishes (78.5 cm^2) (Corning) at a density of 5×10^4 cells/cm^2 and cultured in Williams' medium E supplemented with 5% fetal bovine serum, 10 nM dexamethasone, 10 nM insulin and 100 μg/ml of gentamicin. The medium was changed to fresh medium after 4 h and the cultures were incubated for a further 12 h. The cultures were washed three times with PBS and once with Dulbecco's modified Eagle's medium containing 0.25% gelatin (binding medium), then incubated in binding medium for 1 h at 37°C.

2.3. *Determination of internalized and degraded HGF*

Hepatocytes plated in 6-well collagen-coated plastic dishes (Corning) were incubated in binding medium containing 100 pM [^{125}I]HGF at 4 and 37°C with gentle shaking. Thereafter, the cultures were washed twice with chilled binding medium and three times with ice-cold PBS. To remove the surface-bound HGF from the receptors, the cultures were washed twice with chilled 2 M NaCl in 20 mM HEPES, pH 7.5, then once with 0.2 M glycine-HCl, pH 2.8 containing 140 mM NaCl. The cells were dissolved in 1 ml of a solution containing 2% SDS, 2 mM EDTA and 10 mM $NaHCO_3$ (lysis buffer). The radioactivity was then measured in a γ-counter (Packard). The specific binding was calculated after subtracting the radioactivity of the samples containing a 2,000-fold excess of unlabeled HGF. To analyze the kinetics of HGF-internalization and degradation, hepatocytes were incubated in binding medium containing 100 pM [^{125}I]HGF for 4 h at 4°C with gentle shaking, then washed four times with chilled binding medium. The [^{125}I]HGF bound cells were incubated in binding medium at 37 and 4°C for indicated periods. The culture supernatant was harvested, then the culture was washed with high-salt and acid buffers to remove the surface-bound [^{125}I]HGF. The washed cells were dissolved in lysis buffer. The radioactivity in the culture supernatant, high-salt and acid buffer, as well as the washed cell fraction was measured. To examine the degradation of HGF, each fraction was further incubated with 10% TCA in the presence of 200 μg/ml BSA for 2 h at 4°C. The radioactivity in the TCA-soluble and -insoluble materials was measured separately.

2.4. *Immunoprecipitation of [^{125}I]HGF cross-linked to c-*Met *protein, using anti-*Met *antiserum*

Hepatocytes plated in collagen-coated plastic dishes (78.5 cm^2) (Corning) were incubated in binding medium containing 100 pM [^{125}I]HGF for 4 h at 4°C with gentle shaking. They were then washed four times with chilled binding medium. The [^{125}I]HGF bound cells were incubated in binding medium at 37°C for the indicated periods. They were then washed four times with chilled PBS and incubated with 100 μg/ml BS^3 in 5 ml of 10 mM sodium phosphate, pH 8.3, containing 140 mM NaCl and 1 mM $MgCl_2$ for 30 min at 4°C. The reaction was terminated by adding 5 ml of 25 mM Tris-HCl, pH 7.4, 140 mM NaCl and 1 mM EDTA. The cells were recovered using a scraper and lysed in 400 μl lysis buffer (50 mM Tris-HCl, pH 7.4, containing 140 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A and 100 μg/ml soybean trypsin inhibitor) on ice for 1 h. The lysate was centrifuged at 12,000 × g for 30 min at 4°C. The lysate supernatant was immunoprecipitated with 5 μl anti-*Met* antiserum coupled to 20 μl protein-A Sepharose and the precipitated fraction was separated by SDS/PAGE on 6% polyacrylamide gels under nonreducing conditions. Cross-linked [^{125}I]HGF complexes were detected by autoradiography.

3. RESULTS

3.1. *Metabolism of HGF after the incubation with hepatocytes*

To analyze the metabolism of HGF, [^{125}I]HGF was incubated with cultured hepatocytes, and amount of degraded HGF in the culture supernatant was measured by solubilization with 10% TCA. When [^{125}I]HGF was incubated with the cells at 37°C, the amount of degraded HGF (TCA-soluble) was increased in the culture supernatant (Fig. 1). At 4°C, the degradation was markedly reduced. Because heparin represses more than 80% of HGF-binding to hepatocytes and the c-*Met* protein (Naka et al., in preparation), the effect of heparin on the HGF-degradation was studied. In the presence

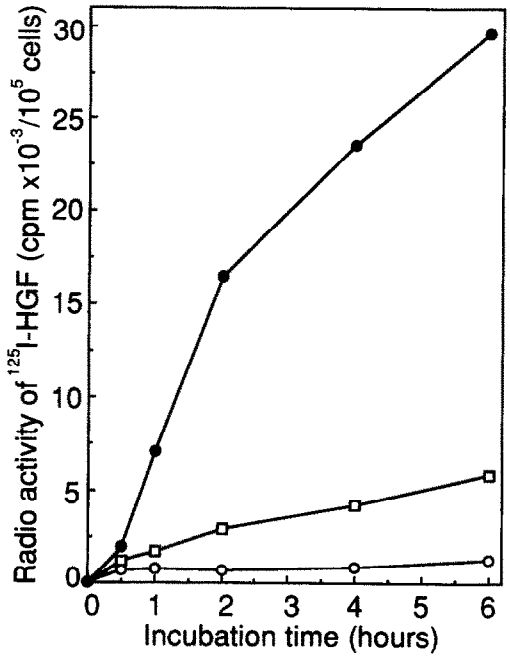

Fig. 1. Degradation of [^{125}I]HGF by cultured hepatocytes. Cells were incubated with 100 pM [^{125}I]HGF at 37°C (●) and 4°C (○) for the indicated periods. The culture supernatant were treated with 10% TCA in the presence of 200 μg/ml BSA for 2 h at 4°C. The TCA-soluble radioactivity was measured. Heparin (100 μg/ml) was added to the culture at 37°C (□).

of heparin, more than 80% of the total degradation was repressed at 37°C.

We then examine whether the degradation of HGF is mediated by receptors on the cells. Fig. 2 shows the time course of HGF-binding using hepatocytes. To remove the bound HGF from the low- and high-affinity receptors, the HGF-bound cells were washed with high-salt and acid buffers (as described in section 2). When [^{125}I]HGF was incubated with hepatocytes at 4°C, the HGF-binding to the cell-surface reached equilibrium at 4 h. After the incubation at 4°C, more than 95% of the bound HGF was released from the cell-surface by high-salt and acid. On the other hand, at 37°C, the amount of [^{125}I]HGF bound to the cells increased for 1 h and subsequently decreased in a time-dependent manner. After the high-salt and acid washing, more than 40% of the bound HGF was retained in the washed cells. These results suggest that surface-bound HGF was internalized in the cells at 37°C, but retained on the cell-surface at 4°C.

3.2. *Kinetics of HGF-internalization and degradation in hepatocytes*

To examine whether the surface-bound HGF is internalized in hepatocytes, then degraded to a TCA-soluble fragment which is thereafter released from the cells, we analyzed the kinetics of HGF-internalization and degradation. [^{125}I]HGF was incubated with hepatocytes at

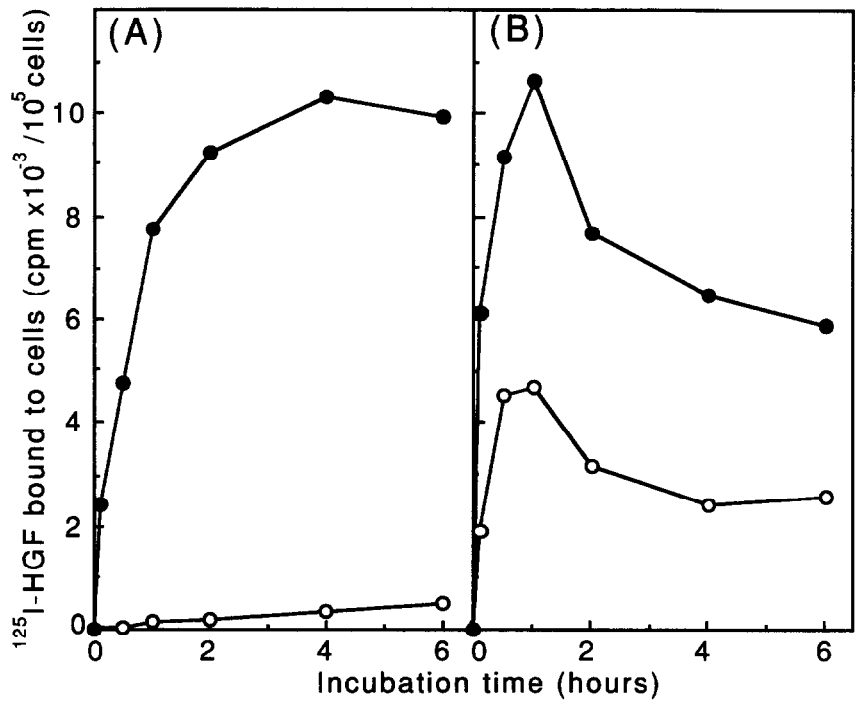

Fig. 2. Time course of HGF-binding to receptors on hepatocytes. Hepatocytes were incubated with 100 pM [^{125}I]HGF at 4°C (A) and 37°C (B) for the indicated periods. To remove surface-bound HGF, the HGF-bound cells (●) were washed with high-salt and acid (as described in section 2) (○). The cells were then extracted and the amount of radioactivity was measured.

4°C for 4 h. To remove free HGF, the culture medium was replaced with fresh medium without [^{125}I]HGF. The HGF-bound cells were incubated at 37 and 4°C. When the cells were incubated at 37°C, the radioactivity level of the surface-bound HGF that was released from the cells by high-salt and acid decreased in a time dependent manner (Fig. 3A). Thereafter, 40% of the surface-bound HGF was rapidly internalized within the first 30 min. After reaching the maximal level, the internalized HGF gradually decreased.

On the other hand, no internalized HGF was detected at 4°C (Fig. 3B). However, the surface-bound HGF was released into the culture supernatant after incubation at 4°C. At 37°C, the amount of radioactive HGF increased in the culture supernatant, and the ratio to the total further increased after 60 min. The HGF-degradation of each fraction, shown in Fig. 3A, was then examined by solubilization with 10% TCA. Most internalized HGF was TCA-insoluble within 30 min, then partially degraded, to become TCA-soluble (Fig. 4A). The radioactivity of surface-bound HGF was completely TCA-insoluble (Fig. 4B). When the total level of internalized HGF decreased after 60 min, the amount of TCA-soluble HGF proportionally increased in the culture supernatant (Fig. 4C). The level was similar to that of internalized HGF. On the other hand, no HGF degradation was observed in the fractions, at 4°C (data not shown). These results indicate that HGF is internalized in hepatocytes at 37°C, degraded and finally released from the cells.

3.3. *Down-regulation of HGF-bound* c-Met *protein on hepatocytes*

We then examined the amount of HGF-bound c-*Met* protein on hepatocytes. To analyze cell-surface HGF/c-*Met* interaction, [^{125}I]HGF cross-linked to c-*Met* protein was immunoprecipitated with a polyclonal antiserum specific to the C-terminal amino acids of murine c-*Met* protein [15]. The resulting complex was analyzed by SDS/PAGE under non-reducing conditions and visualized by autoradiography. As shown in Fig. 5, [^{125}I]HGF cross-linked to c-*Met* protein has an apparent molecular mass of 250 kDa which is that of HGF/c-*Met* complex. Under these conditions, a band with a molecular mass of 70 kDa was that of free HGF. The formation of the [^{125}I]HGF/c-*Met* protein complex was inhibited in the presence of excess unlabeled HGF (data not shown). Thus, HGF is specifically bound to the 160 kDa c-*Met* protein on hepatocytes. After [^{125}I]HGF-bound cells were incubated at 37°C, the rate of formation of the [^{125}I]HGF/c-*Met* protein complex was slightly increased within the first 10 min (Fig. 5, lanes 1–2). Thereafter, the amount of the complex was reduced from the surface in a time dependent manner (Fig. 5, lanes 2–5). These results indicate that HGF-bound c-*Met* protein is down-regulated at 37°C.

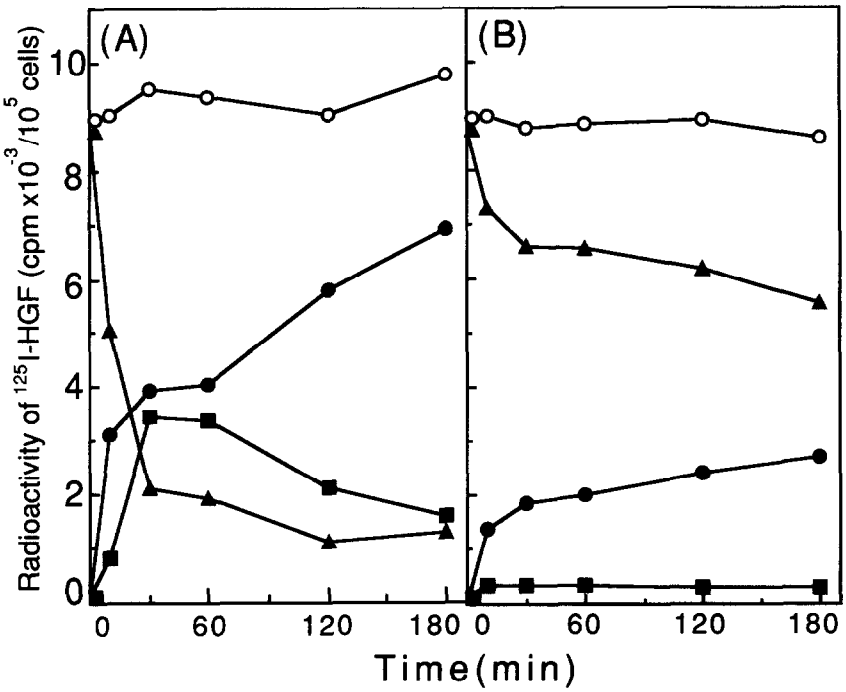

Fig. 3. Kinetic analysis of HGF-internalization. Cells were incubated with 100 pM [^{125}I]HGF at 4°C for 4 h. Thereafter, the culture medium was replaced with fresh binding medium, then incubated at 37°C (A) and 4°C (B) for the indicated periods. The radioactivity in the cultured supernatants (●), high-salt and acid removable fractions (▲) and the unremovable cell fractions (■) was measured. The sum of the counts in these fractions is shown as the total radioactivity (○).

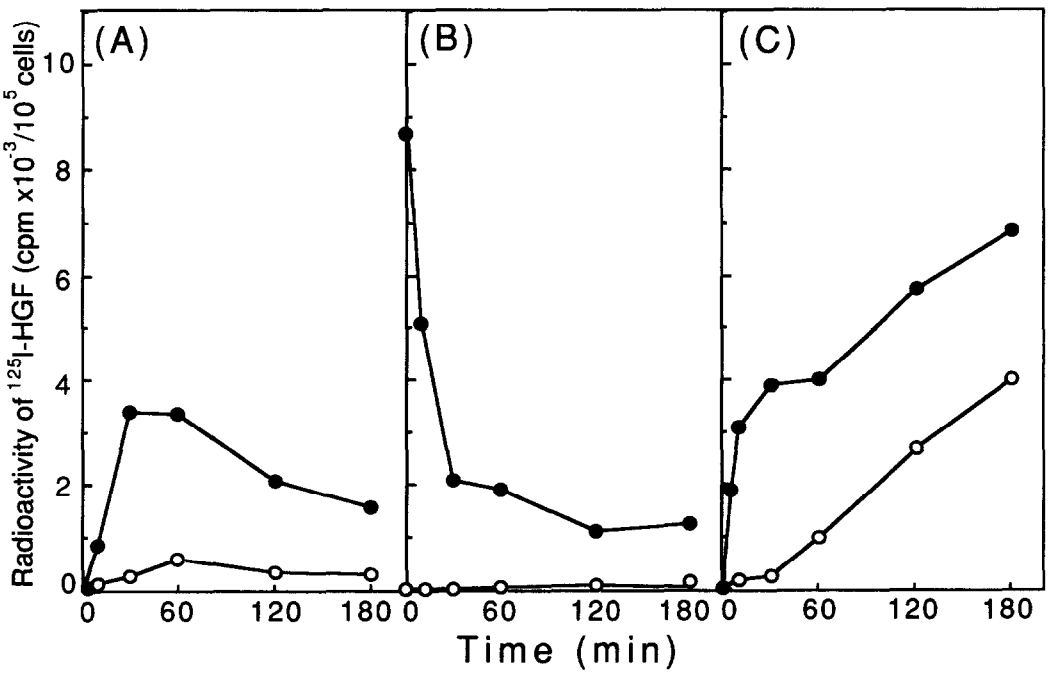

Fig. 4. Kinetic analysis of HGF-degradation. The high-salt and acid resistant fractions (A), the removable fractions (B) and culture supernatant (C) of hepatocytes obtained in the experiment described in Fig. 3A were treated with 10% TCA in the presence of 200 μg/ml BSA for 2 h at 4°C. The TCA-soluble (○) and total (TCA-soluble and TCA- insoluble) (●) radioactivity was measured.

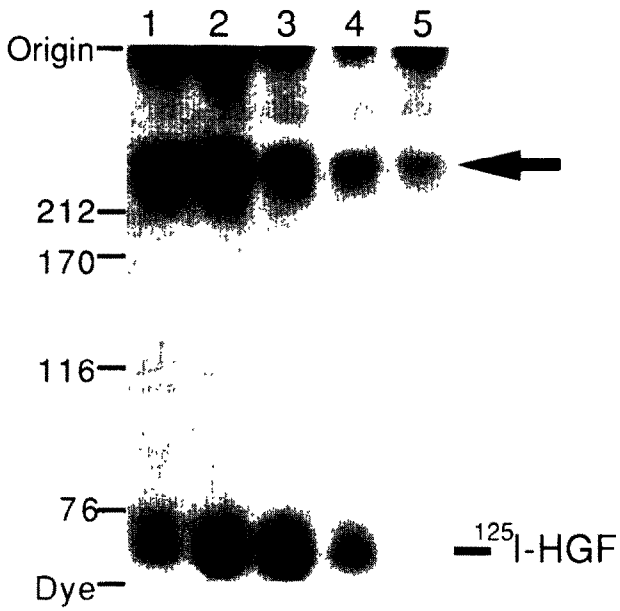

Fig. 5. Down-regulation of HGF bound to c-*Met* on hepatocytes after incubation at 37°C. Cells were incubated with 100 pM [^{125}I]HGF at 4°C for 4 h. Thereafter, the culture medium was replaced with fresh binding medium, then incubated at 37°C (lane 1, 0 min; lane 2, 10 min; lane 3, 30 min; lane 4, 60 min; lane 5, 180 min). The cells were treated with the cross-linker BS^3, lysed and immunoprecipitated with anti-*Met* antiserum. Immunoprecipitates were separated by SDS/PAGE under nonreducing conditions on a 6% polyacrylamide gel and analyzed by autoradiography. The arrow indicates the position of the HGF/c-*Met* protein complex. Molecular mass standards were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa) and transferrin (76 kDa).

4. DISCUSSION

HGF interacts with the cell-surface receptors including c-*Met* protein and heparin-like molecules [13–17]. Typical heparin-binding growth factors including HGF are released from cell-surface heparin-like molecules by high-salt or heparin washing. Moreover, they are released from the high-affinity receptor by acid [13,17,25]. We showed here that cell-surface bound HGF was only partially high-salt and acid removable from hepatocytes at 37°C, but completely removed at 4°C. These results indicate that the residual bound HGF is distinguished from receptor-bound HGF. Thus, the high-salt and acid unremovable HGF would be internalized in the cells. A kinetic study indicated that 40% of the bound HGF was internalized in hepatocytes, degraded and released from the cells. The other bound HGF would be released intact into the culture supernatant from the cell-surface. The $t_{1/2}$ of HGF internalization was about <15 min, and the degradation was estimated as occurring within 100–120 min.

Rapid internalization and degradation of ligands has been reported [20,21]. The $t_{1/2}$ of IL-2 internalization is about 10 min and the degradation is 60–80 min in human T cells. IL-2 internalization is mediated by the high-affinity receptor. The c-*Met* protein binds HGF with high-affinity, and the K_d value is in the 10^{-11} M range [14,15]. When surface-bound HGF was internalized in hepatocytes, HGF-bound c-*Met* protein proportionally decreased from the surface. Thus, some HGF-internalization is mediated by the c-*Met* protein. On the other hand, HGF has affinity for heparin-like molecules, commonly found as heparan sulfate proteoglycan (HSPG) at the cell-surface and the extracellular matrix [13–15,17]. Scatchard analysis of HGF has revealed that the target cells, including hepatocytes, possess a small number ($\sim 10^3$ per cell) of the high affinity receptor/c-*Met* protein and a large number ($\sim 10^7$ per cell) of the low affinity receptor/HSPG [14,15]. Our results showed that 40% of surface-bound HGF was internalized and degraded in hepatocytes. In some cell lines, bound HGF was completely internalized, then degraded (data not shown). However, the ratio of internalized HGF to surface-bound HGF is too high to explain the HGF-internalization mediated by a small amount of c-*Met* protein. In addition, cell-associated HSPG, which can bind heparin-binding growth factors, is rapidly internalized and degraded in a variety of cells [26,27]. For example, internalization of basic FGF, a heparin-binding growth factor, is mediated by the high- and low-affinity receptors [28]. Thus, HGF may be internalized through two pathways mediated by the high-affinity receptor/c-*Met* protein and the low-affinity receptor/HSPG. It is of note that cell-surface HSPG accumulates in the nucleus and regulates the growth of a hepatoma cell line [29]. Further study will elucidate the HGF-signal transduction mediated by each pathway.

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