Production of Functional Hepatocyte Growth Factor (HGF) in Insect Cells Infected with an HGF-Recombinant Baculovirus in a Serum-Free Medium

ARTICLE *in* BIOTECHNOLOGY PROGRESS · DECEMBER 1999

Impact Factor: 2.15 · DOI: 10.1021/bp990143t · Source: PubMed

4 AUTHORS, INCLUDING:

M.-Y. Wang
National Chung Hsing University
45 PUBLICATIONS **550** CITATIONS

SEE PROFILE

Production of Functional Hepatocyte Growth Factor (HGF) in Insect Cells Infected with an HGF-Recombinant Baculovirus in a Serum-Free Medium

Min-Ying Wang,*,† Ya-Huey Yang,† Hsuan-Shu Lee,‡ and Su-Yuan Lai§,||

Graduate Institute of Agricultural Biotechnology, National Chung Hsing University, Taichung, Taiwan 40227 ROC, and Department of Internal Medicine and Institute of Biochemical Science, National Taiwan University, Taipei, Taiwan

Three insect cell lines, SL-7B cells derived from *Spodoptera litura*, Sf9, and High Five (Hi-5) cells, were used for the production of pro-hepatocyte growth factor (pro-HGF). Cells were cultured and then infected with a recombinant HGF-containing baculovirus in a serum-free medium. In SL-7B cells, pro-HGF is synthesized and excreted from the cells and late in infection is converted to a heterodimeric form of HGF even when the cells are grown in serum free medium. Conversion of a single-chain form of HGF (pro-HGF) into an HGF heterodimer was unexpected, as pro-HGF is normally cleaved by a serum protease called HGF activator. The proliferation activity of heparin-affinity-purified HGF from serum-free culture supernatant of SL-7B cells is comparable to that obtained from HGF converted by serum proteases, suggesting that SL-7B cells produce a functionally analogous protease to correctly process pro-HGF. This work reports, for the first time, on the feasibility of properly processing pro-HGF to form functional HGF by proteases from invertebrate cells in serum-free media. Avoiding the supplementation of sera provides the advantages of a low production cost, zero contamination of infectious agents from sera, and simple downstream product purification. Experimental results further demonstrate that the conversion of pro-HGF by insect cells is cell-line-dependent, because proteases in Hi-5 or Sf9 cells could not process pro-HGF as efficiently and properly as those in SL-7B cells.

Introduction

Hepatocyte growth factor (HGF) had been purified from the serum of hepatectomized rats (*1*−*2*), as a potent mitogen for adult rat hepatocytes in primary culture (*3*−*5*). Subsequent investigations revealed that HGF could stimulate the growth of a variety of cell types, including melanocytes, keratinocytes, renal tubular cells, breast carcinoma cells, and endothelial cells (*6*−*10*). Moreover, a related study confirmed the ability of HGF, also known as a scatter factor, to dissociate or induce morphogenesis of epithelial cells in culture (*11, 12*). Therefore, HGF may significantly contribute to embryogenesis, wound repair, or even tumor invasion.

HGF is first synthesized and secreted as an inactive single-chain precursor protein (pro-HGF) with a molecular weight of 91 kDa (*9, 13*−*16*). This precursor is further proteolytically cleaved between Arg494 and Val495 to form a heterodimer composed of a 69-kDa α-chain and a 34-kDa *β*-chain held together by disulfide bonds (*14, 17*). The proteolytic cleavage of pro-HGF is a prerequisite for the biological activity of HGF (*18*−*20*). Related investigations have identified the protease responsible for cleavage as the HGF activator, which was purified from fetal bovine serum (FBS) (*21*) or human serum (*22*). This purified HGF activator has a molecular mass of 34 kDa, consisting of two peptide chains linked together by a disulfide bond (*22*). Another HGF-converting enzyme purified from FBS has a molecular weight of 90 kDa (*23*). However, the relationship between the HGF activator and the HGF converting enzyme is unclear (*23*).

The studies of the physiological role of HGF in vivo and the effects of administering exogenous HGF to animals have been hampered by the lack of large quantities of HGF. Miyazawa et al. (*13*) and Nakamura et al. (*14*) investigated the overexpression of recombinant HGF in COS cells but the production level is low. Attempts to obtain HGF using an insect cells/baculovirus expression system were also limited with low yields (approximately, 1 mg/L), although the yields were higher than those expressed in the mammalian cell system (*24, 25*). Furthermore, as in the mammalian cell system, recombinant HGF is converted from the unprocessed single-chain pro-HGF by a protease, which is presumably present in fetal bovine serum, after the infected cells synthesize pro-HGF and secrete it into culture media (*24*−*26*). The supplementation of a serum in a culture medium makes the purification of HGF more complicated and requires more purification steps to remove the undesired serum proteins. A strategy to avoid the use of a serum is to produce pro-HGF in a serum-free medium and then convert it to functional HGF by a protease. To achieve our goal, three

* Tel: 886-4-285-6697. Fax: 886-4-285-6697, 886-4-285-3527. E-mail: mywang@dragon.nchu.edu.tw.
† National Chung Hsing University.
‡ Department of Internal Medicine, National Taiwan University.
§ Institute of Biochemical Science, National Taiwan University.
|| Current address: Department of Food Health, Chungtai Institute of Health Sciences and Technology, #11, Pu-tzu Lane, Pei-tun District, Taichung, Taiwan, 40605.

10.1021/bp990143t CCC: $19.00
Published on Web 02/24/2000

insect cell lines, SL-7B cells derived from *Spodoptera litura*, Sf9, and High Five (Hi-5) cells, were evaluated for the production of pro-HGF. This study demonstrates that the recombinant pro-HGF produced from certain insect cells, such as SL-7B cells, can be proteolytically converted into active HGF in a serum-free medium. This finding facilitates not only the production but also the purification of HGF. For the production of a therapeutic agent such as HGF, avoiding the supplementation of serum lowers the production cost and reduces the potential risk of contamination by mycoplasma and infectious viruses. From the viewpoint of purification, in contrast to the method used to purify the extracellular proteins in conventional serum-containing media, the purification steps of HGF in serum-free media are less tedious and more efficient.

The baculovirus expression system is extensively applied to produce eukaryotic proteins and is a highly effective system for the expression of a foreign gene (*27*). In this system, *S. frugiperda* cells, mainly Sf9 and Sf21 cells, are commonly used as the host cells for studying the effects of viral and /or cellular proteases on the expressed recombinant proteins (*28*−*32*). However, other cell lines have seldom been investigated. A commercially available insect cell line such as the High Five cell line (Hi-5 cells) supports a high level of recombinant protein production and has been used to produce vaccine candidates (*33*). Another cell line, SL-7B cells, was derived from *S. litura*; our laboratory is currently evaluating it for its potential as host cells to produce HGF. Among the several advantages of applying this cell line in recombinant protein production by using a baculovirus expression system include its stability for in vitro culture, easy maintenance in serum-free media, and excellent growth of the cells in suspension (*34*). Therefore, this study examines the proteases in these particular cell lines and their effect on recombinant protein integrity expressed by using the baculovirus expression system. In addition, HGF is used as a model protein to examine the pattern of proteolytic degradation of pro-HGF by the three cell lines, Sf-9, Hi-5 and SL-7B cells.

Methods and Materials

Cell Lines and Cultures. *S. litura* (SL-7B), *S. frugiperda* (Sf9), and *Trichoplusia ni* (Hi-5) cells were maintained in T-flasks as described elsewhere (*35*) with TNM-FH media (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS, GIBCOBRL, Gaithersburg, MD) at 27 °C in an incubator. Cells were adapted to EX-CELL 401 serum-free medium (JRH Biosciences, Lenexa, KS) and then maintained in shaking flasks in a shaker. Viable cells were counted after being stained with a 0.2% trypan blue dye solution. Cells were then seeded at a density of 3×10^5 cells/mL in 20 mL of growth media in 200 mL shaking flasks and incubated at 27 °C on an orbital platform shaker at a speed of 250 rpm. BN CL.2 (mouse hepatocytes) cells were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCOBRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS, GIBCOBRL, Gaithersburg, MD). The culture was maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Generation of Recombinant Viruses and Preparation of Serum-Free Virus Solution. The recombinant baculovirus HGF-pVL 1393 was generated by homologous recombination and plaque purification as described by Lee et al. (*24*). The virus was propagated by infecting Sf9 cells in a 6-well plate and then amplified in T-25 flasks by using TNM-FH medium supplemented with 10% fetal bovine serum. Adopting the following method generated serum-free virus stock. Sf9 cells cultured in EX-CELL 401 medium were seeded in several T-75 flasks (Corning, NY) at a density of 1×10^6 cells/mL, and 200 μL of serum-containing HGF-pVL 1393 viral solution was added. After 2 h of viral infection, the culture medium was discarded, and the cells were washed with 5 mL of fresh EX-CELL 401 serum-free medium three times. Finally, 15 mL of fresh EX-CELL 401 serum-free medium was poured into each flask for viral replication. After 5 days, the culture supernatant was collected and used as a serum-free virus stock solution. We prepared approximately 300 mL of the virus stock solution for the use in all experiments. Finally, the virus titer was determined by the end-point dilution method (*27*).

Expression, Partial Purification, and Determination of HGF Concentrations. To express the recombinant protein pro-HGF, the amplified serum-free recombinant viruses were used to infect Sf9, Hi-5, or SL-7B cells cultured with EX-CELL 401 expression medium in a shaker. The conditions of cell culture were described earlier. Next, cells were infected with a multiplicity of infection (MOI) of 0.1 by adding serum-free recombinant HGF-pVL 1393 virus solution. Samples (1 mL) were taken daily for cell number counting and Western blotting analysis. For the bench-scale production and purification of recombinant HGF, SL-7B cells were seeded at a density of 5×10^5 viable cells/mL in 250 mL of growth media in a 500 mL spinner flask and incubated at 27 °C on a magnetic stirrer at a speed of 150 rpm. Samples (3 mL) were taken daily for viable cell counting, pH measurement, and Western blotting analysis. After 11 days post-infection, the remaining culture was collected and centrifuged at 12000*g* for 20 min. The HGF-containing supernatant was used for protein purification after NaCl was added to a final concentration of 0.4 M. A total of 200 mL of supernatant was filtered on a 0.45 μm membrane filter (Millipore, Bedford, MA) by vacuum suction, cooled to 4 °C, and then chromatographed in a heparin-agarose column (bed volume 12.5 mL), which was earlier equilibrated with 75 mL of phosphate-buffered saline (PBS) buffer containing 0.4 M NaCl. After binding the proteins to the resin, 75 mL of 0.4 M NaCl in PBS buffer was used to wash the column to remove the unbound molecules. A higher NaCl concentration (0.6 M, 75 mL) of PBS buffer was used to remove more impurity from resin. The recombinant HGF was then eluted with 75 mL of PBS containing 1.2 M NaCl. Fractions containing HGF were collected and concentrated by using a Ultrafree-15 centrifugal filtration device with a nominal molecular weight limit of 10 kDa (Millipore, Bedford, MA). Finally, HGF concentration was determined by the ELISA method (*24*).

Western Blotting Analysis of the Baculovirus-Expressed HGF. Samples, including a culture supernatant from noninfected cells (as a control) or from HGF-expressing cells (Sf9, Hi-5, or SL-7B cells), were mixed with sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 min, and then resolved on a 10% SDS−polyacrylamide gel electrophoresis (SDS−PAGE) gel. The proteins were transferred onto a PVDF membrane. After being blocked in 5% nonfat milk (DIFCO LABORATORIES, Detroit, MI), the membrane was incubated for 1 h at room temperature with HGF antiserum (either α-chain or β-chain specific monoclonal antibody), which was prepared according to Lee et al. (*24*), and diluted 2000× in 5% nonfat milk containing 0.1% Tween-20. The

membrane was then washed three times in PBS buffer containing 0.1% Tween-20, incubated for 0.5 h with alkaline phosphatase conjugated goat anti-mouse IgG antibody (Jackson ImmunoReasearch, West Grove, PA) diluted 10 000× in 5% nonfat milk containing 0.1% Tween-20. After washing, the membrane was color-developed in 10 mL of alkaline phosphatase buffer containing 66 μL of NBT and 33 μL of BCIP stock solution prepared as described in Sambrook et al. (*36*).

Proliferation Activity Assay. BN CL.2 cells were seeded on 96-well culture plates (Corning, NY) at a density of 10^4 cells/100μL/well in the medium of DMEM containing 10% of FCS. After the cells cultured overnight, the medium was changed to DMEM serum-free medium. Ten microliters of PBS containing 0, 0.1, 0.5, 1, 1.5, 3, 4, 6, and 8 ng, respectively, of the concentrated purified HGF were added 1 day later into triplicate wells. Two days after the addition of HGF, the viable cell numbers were determined by a colorimetric method according to the instruction manual provided in a CellTiter 96 Aqueous Assay kit (Promega Corporation, Madison, WI). Briefly, absorbance at 492 nm was determined on an ELISA reader 3 h after adding the combined MTS/PMS solution. The increase in absorbance is directly proportional to the number of viable cells, as MTS is converted into a colored product by the mitochondria of living cells.

Results and Discussion

Proteolytic Processing of Pro-HGF by SL-7B Cells in a Serum-Free Medium. Our previous study demonstrated that the functional two-chain form of recombinant HGF can be expressed by Sf9 cells or Hi-5 cells infected with a recombinant baculovirus HGF-pVL 1393 when the cells were cultured in serum-containing media (*24*). To test the production level of pro-HGF in SL-7B cells, which is a local isolate, we grew the cells in EX-CELL 401 serum-free medium and then infected them by adding the serum-free HGF-pVL 1393 virus solution. Under these circumstances, the infected cells presumably only produce pro-HGF. However, surprisingly, the two-chain form of HGF presented in the culture supernatant. The conversion of pro-HGF to the two-chain form of HGF was unexpected under our experimental conditions because the culture medium was not supplemented with FBS, which contains the HGF activator or HGF converting enzyme, a protease responsible for pro-HGF cleavage. Thus, to thoroughly investigate the proteolytic processing of pro-HGF and provide further insight into the dynamics of the formation of the two-chain form of HGF, experiments involving viral infection of SL-7B cells were performed in the shaking and spinner flasks. Figure 1 displays the time course of pro-HGF processing and the formation of the α-chain of HGF. Pro-HGF was first synthesized and secreted into the culture medium from day 1 post-infection (lane 1, Figure 1). The yield of the secreted pro-HGF was very stable from day 1 to 7 post-infection, whereas the amount of the α-chain of HGF steadily increased during this period (lanes 2−4, Figure 1). The viability of the cells declined to about 10% at day 7 post-infection; meanwhile, the synthesis rate of pro-HGF might be slower than that of the degradation of pro-HGF. Therefore, all of the pro-HGF was expected to be converted into a two-chain form of HGF 2 days later (i.e., day 9 post-infection). To ensure that all of the pro-HGF was completely converted into a two-chain form of HGF, the culture medium was harvested after day 11. Therefore, only α-chain of HGF derived from cells harvested at day 11 were clearly visualized on the Western blot shown in Figure 1 (lane 6). The β-chain of HGF was also detected using a monoclonal antibody against the β-chain of HGF on the Western blot (data not shown). As expected, pro-HGF has been completely processed and was not detectable in the blot (data not shown). In contrast, the controls, in which cells were harvested 3 days after viral infection, indicated that pro-HGF was not completely converted into a two-chain form of HGF (Figure 1, lane C). The results in Figure 1 indicate that a single-chain form of HGF (pro-HGF) is first secreted and slowly converted to a two-chain form of HGF in a serum-free medium when SL-7B cells were infected with HGF-pVL 1393 recombinant baculoviruses.

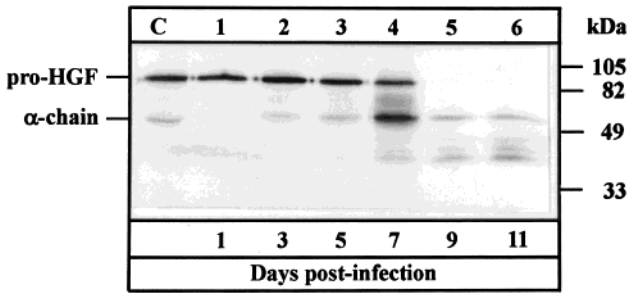

Figure 1. Activation of pro-HGF to the heterodimeric form of HGF by the SL-7B cells cultured in shaking flasks and infected with a recombinant baculovirus, HGF-pVL 1393, in a serum-free medium. Western blot analysis was performed by separating samples on SDS−PAGE, blotting onto a PVDF membrane, and detecting with a monoclonal antibody against the α-chain of HGF. Molecular weight markers are indicated on the right-hand side of the gel. Lane C was loaded with a mixture of pro-HGF and the two-chain form of HGF as a control. Lanes 1−6 were loaded with 20 μL of culture supernatant at days 1, 3, 5, 7, 9, and 11 post-infection, respectively.

Production and Partial Purification of Two-Chain Form of HGF. The above experiment has already demonstrated that the SL-7B cells might produce proteases to process pro-HGF to a two-chain form of HGF in a serum-free medium. The next step was the bench-scale production and purification of HGF. In this manner, a sufficient amount of HGF could be obtained for a proliferation activity assay to further confirm the proper processing of pro-HGF in SL-7B cells. A 500-mL spinner flask with a 200-mL working volume was used for this purpose (the bench-scale production of HGF). After infection, the viability started to decline and was near 70% at 100 h post-infection (h p.i.). After all cells were stained with trypan blue at 250 h p.i., cell broth was collected and centrifuged. The supernatant was then processed to purify HGF as described in Materials and Methods. According to Figure 2, the most two-chain form of HGF was eluted from the heparin−agarose column when the concentration of NaCl in PBS buffer was stepwisely increased from 0.4 to 1.2 M (lanes 6 and 7 in the top gel for the α-chain of HGF and lane 1 in the bottom gel for the β-chain of HGF). The fractions eluted by PBS buffer containing 1.2 M NaCl were collected and concentrated. Finally, the concentrations of heparin-affinity-purified and membrane-concentrated HGF were measured by ELISA, in which the production level for HGF was estimated to be 60 μg/L.

Proliferation Activity Assay of Heparin-Affinity-Purified HGF Produced by SL-7B Cells. The function of HGF, converted by proteases in SL-7B cells and partially purified using heparin affinity chromatography, was assessed by determining its ability to stimulate the growth of BN CL 2 cells. The cells, differentiated from mouse hepatocytes, have shown a proliferation response to HGF and were used in the proliferation activity assay instead of labor-intensive primary hepatocyte cultures

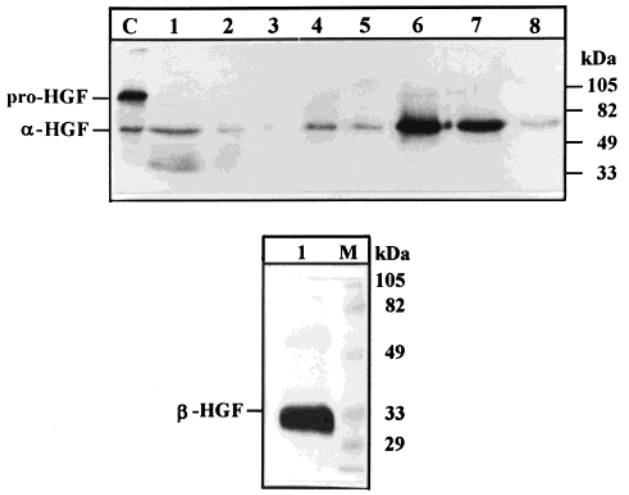

Figure 2. HGF purification using a heparin-affinity chromatography column. The top gel shows the Western blotting analysis of fractions collected from a heparin column in PBS buffer containing 0.4−1.2 M NaCl. Protein molecular weight makers in kDa are shown on the right. Lane C was loaded with a mixture of pro-HGF and the two-chain form of HGF as a control. Lane 1 is flow-through. Lanes 2 and 3, 4 and 5, and 6−8 are fractions eluted by PBS buffer containing 0.4, 0.6, and 1.2 M NaCl, respectively. The bottom gel shows the Western blot analysis of the concentrates of the collection of fractions 6−8 shown in the top gel (lane 1). Lane M is the protein MW makers. The size is indicated in kDa on the right.

(*24*). According to Figure 3, although the concentrate of medium from a negatively controlled insect cell culture did not affect the growth of BN CL.2 cells (data not shown), the medium containing the heparin-affinity-purified and concentrated HGF stimulated cell growth in a dose-dependent manner (Figure 3). Furthermore, proliferation activities of various amounts of the concentrated and partially purified HGF on BN CL.2 cells are very close to those obtained from the recombinant HGF expressed in serum-containing media, in which the active two-chain form of HGF dominated (*24, 37*). Therefore, our results strongly indicate that pro-HGF was properly processed to an active two-chain form of HGF by the infected SL-7B cells in a serum-free medium.

Conversion of Pro-HGF by Insect Cells Is Cell Line-Dependent. The conversion of pro-HGF was examined in two other insect cell lines, Hi-5 or Sf9 cells, currently used in the field of baculovirus expression systems, by infecting the cells with the same virus solution at the same MOI as above. According to Figure 4A, the conversion of pro-HGF to α-chain HGF by Hi-5 cells was rare. However, the first degradation product that appeared after 2 days post-infection (d p.i.) was analyzed by using Western blot and was an 82-kDa protein with unknown function. It can be recognized by an antibody against the α-chain of HGF. From 5 d p.i., the 82 kDa protein gradually disappeared, and then two low molecular weight (33−49 kDa) polypeptides formed instead (Figure 4A). However, as shown in Figure 4B, the degraded product pattern from pro-HGF by Sf9 cells markedly differed from that obtained from SL-7B cells or Hi-5 cells. Interestingly, most pro-HGF still remained in the culture medium at 11 d p.i. (Figure 4B). This phenomenon is consistent with the findings of Yee et al. (*25*), in which (a) pro-HGF was predominant in the culture supernatant and (b) only a few pro-HGF proteins were proteolytically degraded to a two-chain form of HGF when the viral infection went beyond 6 days post-infection.

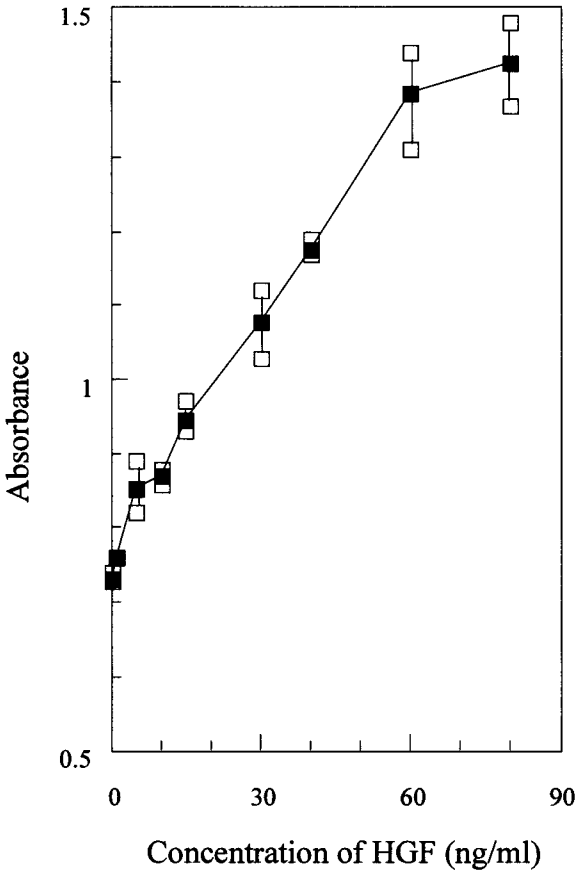

Figure 3. Dose−response effect of the purified HGF on the proliferation of BN CL.2 cells (a mouse hepatocyte cell line). Cells were seeded at a density of 10^4 cells/100μL medium/well in DMEM with 10% FCS on 96-well culture plates and cultured overnight. Following change of the medium to DMEM serum-free, the next day, 10 μL of PBS containing 0, 0.1, 0.5, 1, 1.5, 3, 4, 6, and 8 ng of the concentrated purified HGF was added into triplicate wells, respectively. The viable cell numbers were determined by a colorimetric method after a 2-day incubation. Each value (solid squares) represents the mean ± SD (empty squares) of triplicate measurements.

Previous studies have demonstrated the ability of the proteases encoded by Sf9 cells infected by a recombinant baculovirus to properly process a pro-form of papain, a plant protease. For the overexpression of papain, Vernet et al. (*38*) postulated that an Sf9 cellular protease is involved in the formation of this active cysteine protease. In their investigation, a synthetic gene coding for the precursor of papain was expressed using baculovirus/Sf9 insect cell system (*38*). The precursor of papain was secreted principally as a zymogen, and then it was converted into active papain by unknown cellular factors, which were released into the culture medium from cell lysosome as a result of cell lysis at late stages of infection. However, the protease responsible for the activation of papain cleaved pro-papain at the position of Asn-Ile or Gly-Asp or Asn-Asp, not Arg-Val as in the activation of HGF (*38*). This may account for why Sf9 cells cannot process pro-HGF as efficiently as SL-7B cells. As for Hi-5 cells, they may contain proteases that only degrade pro-HGF nonspecifically. Further work is underway to identify the unique factors involved in the activation of pro-HGF from the infected SL-7B cells. Of pertinent interest to identify and characterize the cellular proteases is to elucidate their importance in processing zymogen protein with significant biological function.

As widely recognized, effectively controlling nonspecific degradation of baculovirus-expressed recombinant pro-

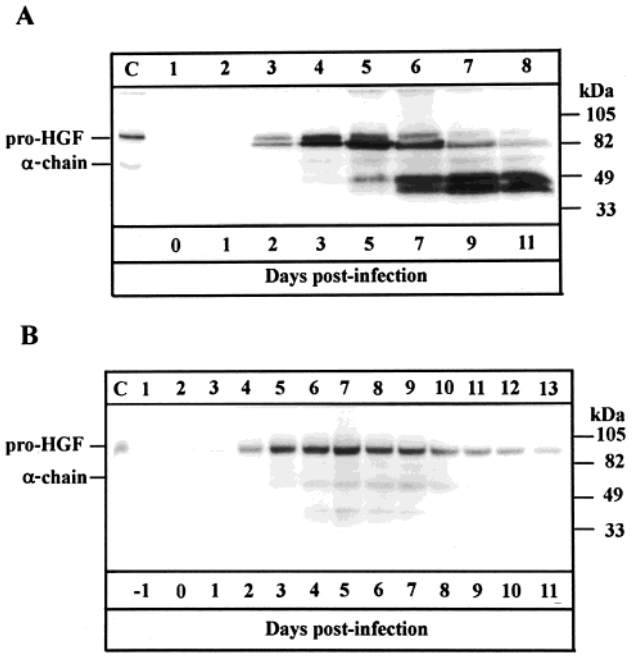

Figure 4. Western blot analysis of the dynamic degradation of pro-HGF by proteases in the infected (A) Sf9 or (B) Hi-5 cells. Sf9 and Hi-5 cells were separately cultured in two 200 mL shaking flasks with 20 mL working volume in the EX-CELL 401 medium and infected by adding the HGF-pVL 1393 serum-free virus solution. In both A and B, lane C is the control as illustrated earlier in Figure 1. In A, lanes 1−8 were loaded with 20 μL of culture supernatant taken at days 0, 1, 2, 3, 5, 7, 9, and 11 post-infection. In B, lanes 1−13 were loaded with 20 μL of culture supernatant taken from 1 day before viral infection to 11 days post-infection.

teins is important. A common practice is to add a cocktail of protease inhibitors to baculovirus-infected insect cell culture when the proteases involved in the degradation of recombinant proteins have not been identified. In this study, we also observed the nonspecific degradation of HGF in the three kinds of cells used herein (SL-7B, Sf9, and Hi-5 cells) when they were infected with the HGF-pVL 1393 recombinant baculovirus generated in EX-CELL 401 medium (Figure 1, lanes 3−6; Figure 4A, lanes 3−8; Figure 4B, lanes 6−12). The nonspecific products may be formed by proteases in response to cell stress and the overexpression of foreign proteins due to viral infection. As mentioned earlier, for Sf9 cells, proteases produced in response to baculovirus infection have been described (*30, 39*). The protease identified from Sf9 cells by Slack et al. (*30*) was a cathepsin L-like proteinase with a molecular weight of 37 kDa, which was encoded by a gene in AcMNPV genome. The biological function of this protease was linked to the degradation of a host tissue. A recent investigation found three acidic proteases with molecular weights of 49, 40, and 36 kDa, respectively, in Sf9 cells infected with a recombinant baculovirus, v-IBD7, in which target proteins (VP2, VP3, and VP4 structural proteins of an infectious bursal disease virus) and a reporter protein, β-galactosidase (β-gal), were expressed (*32*). The most abundant protease of 49 kDa found in Sf9 cell extract (cysteine protease activity) could degrade gelatin, β-gal, and bovine serum albumin. Two other proteases (40 and 36 kDa) appeared after 72 h p.i. There have been no reports concerning the specific activity of these or other proteases toward the degradation or activation of pro-HGF. We are currently focusing on the identification of the unique cellular proteases involved in HGF activation in SL-7B cells and the mechanism of the formation of the protease. Our upcoming work will also determine whether the proteases found in Sf9 cells are involved in the activation or nonspecific degradation of pro-HGF.

Acknowledgment

This work was supported in part by National Science Council (grant NSC 87-2313-B-005-081), Taipei, Taiwan. Dr. Su-Yuan Lai was a postdoctoral fellow under grant NSC-87-2312-B-002-015-B15 awarded to Prof. Shyh-Horng Chiou of the Institute of Biochemical Science, National Taiwan University (NTU) from the National Science Council. The SL-7B cells were kindly provided by Dr. Chung-Hsiung Wang (Department of Entomology, NTU, Taipei, Taiwan). We also thank Prof. William E. Bentley (Department of Chemical Enineering, University of Maryland, College Park, MD) and Prof. Shih-Hsiung Wu (Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan) for critical review of the manuscript and Ms. Ling-Ling Chiou (Emergency Medicine, NTU, Taipei, Taiwan) for technical assistance.

References and Notes

(1) Michalopoulos, G. K.; Houck, K. A.; Dolan, M. L.; Luetteke, N C. Control of hepatocyte proliferation by two serum factors. *Cancer Res.* **1984**, *44*, 4414−4419.

(2) Nakamura, T.; Nawa, K.; Ichihara, A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 1450−1459.

(3) Nakamura, T.; Nawa, K.; Ichihara, A.; Kaise, N.; Nishino, T. Purification and subunit structure of hepatocyte growth factor from rat platelets. *FEBS Lett.* **1987**, *224*, 311−316.

(4) Gohda, E.; Tsubouchi, H.; Nakayama, H.; Hirono, S.; Sakiyama, O.; Takahashi, K.; Miyazaki, H.; Hashimoto, S.; Daikuhara, Y. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. *J. Clin. Invest.* **1988**, *81*, 414−419.

(5) Zarnegar, R.; Michalopoulos, G. K. Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocyte. *Cancer Res.* **1989**, *49*, 3314−3320.

(6) Kan, M.; Zhang, G. H.; Zarnegar, R.; Michalopoulos, G.; Myoken, Y.; McKeehan, W. L.; Stevens, J. I. Hepatocyte growth factor/hepatopoietin A stimulates the growth of rat kidney proximal tubule epithelial cells (RPTE), rat nonparenchymal liver cells, human melanoma cells, mouse keratinocytes and stimulates anchorage-independent growth of SV-40 transformed RPTE. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 331−337.

(7) Igawa, T.; Kanda, S.; Kanetake, H.; Saitoh, Y.; Ichihara, A.; Tomita, Y.; Nakamura T. Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. *Biochem. Biophys. Res. Commun.* **1991**, *174*, 831−838.

(8) Matsumoto, K.; Tajima, H.; Nakamura, T. Hepatocyte growth factor is a potent stimulator of human melanocyte DNA synthesis and growth. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 45−51.

(9) Rubin, J. S.; Chan, A. M.; Bottaro, D. P.; Burgess, W. H.; Taylor, W. G.; Cech, A. C.; Hirschfield, D. W.; Wong, J.; Miki, T.; Finch, P. W. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 415−419.

(10) Grant, D. S.; Kleinman, H. K.; Goldberg, I. D.; Bhargava, M. M.; Nickoloff, B. J.; Kinsella, J. L.; Polverini, P.; Rosen, E. M. Scatter factor induces blood vessel formation in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1937−1941.

(11) Montesano, R.; Matsumoto, K.; Nakamura, T.; Orci, L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **1991**, *67*, 901−908.

(12) Weidner, K. M.; Arakaki, N.; Hartmann, G.; Vandekerckhove, J.; Weingart, S.; Rieder, H.; Fonatsch, C.; Tsubouchi, H.; Hishida, T.; Daikuhara, Y. Evidence for the identity of

human scatter factor and human hepatocyte growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7001−7005.
(13) Miyazawa, K.; Tsubouchi, H.; Naka, D.; Takahashi, K.; Okigaki, M.; Arakaki, N.;Nakayama, H.; Hirono, S.; Sakiyama, O.; Takahashi, K.; Kitamura, N. Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. *Biochem. Biophys. Res. Commun.* **1989**, *163*, 967−973.
(14) Nakamura, T.; Nishizawa, T.; Hagiya, M.; Seki, T.; Shimonishi, M.; Sugimura, A.; Tashiro, K.; Shimizu, S. Molecular cloning and expression of human hepatocyte growth factor. *Nature* **1989**, *342*, 440−443.
(15) Miyazawa, K.; Kitamura, A.; Naka, D.; Kitamura, N. An alternatively processed mRNA generated from human hepatocyte growth factor gene. *Eur. J. Biochem.* **1991**, *197*, 15−22.
(16) Hernandez, J. R.; Michalopoulos, G. K. Characterization of the effects of human placental HGF on rat hepatocytes. *J. Cell. Physiol.* **1992**, *150*, 116−121.
(17) Naka, D.; Ishii, T.; Yoshiyama, Y.; Miyazawa, K.; Hara, H.; Hishida, T.; Kitamura, N. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. *J. Biol. Chem.* **1992**, *267*, 20114−20119.
(18) Gak, E.; Taylor, W. G.; Chan, A. M. L.; Rubin, J. S. Processing of hepatocyte growth factor to the heterodimeric form is required for biological activity. *FEBS Lett.* **1992**, *311*, 17−21.
(19) Hartmann, G.; Naldini, L.; Weidner, K. M.; Sachs, M.; Vigna, E.; Comoglio, P. M.; Birchmeier, W. A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the *c-Met* receptor and induces cell dissociation but not mitogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11574−11578.
(20) Lokker, N. A.; Mark, M. R.; Luis, E. A.; Bennett, G. L.; Robbins, K. A.; Baker, J. B.; Godowski, P. J. Structure−function analysis of hepatocyte growth factor: identification of variants of that lack mitogenic activity yet retain high affinity receptor binding. *EMBO J.* **1992**, *11*, 2503−2510.
(21) Shimomura, T.; Ochiai, M.; Kondo, J.; Morimoto, Y. A novel protease obtained from FBS-containing culture supernatant that processes single chain form hepatocyte growth factor to two chain form in serum-free culture. *Cytotechnology* **1992**, *8*, 219−229.
(22) Miyazawa, K.; Shimomura, T.; Kitamura. A.; Kondo, J.; Morimoto, Y.; Kitamura, N. Activation of the zymogen of hepatocyte growth factor activator by thrombin. *J. Biol. Chem.* **1993**, *268*, 10024−10028.
(23) Mizuno, K.; Tanone, Y.; Okano, I.; Harano, T.; Takada, K.; Nakamura, T. Purification and characterization of hepatocyte growth factor (HGF)-converting enzyme: activation of pro-HGF. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 1161−1169.
(24) Lee, H. S.; Huang, G. T.; Sheu, J. C.; Chiou, L. L.; Lai, M. Y.; Chen, D. S.; Lee, S. C. Characterization of hepatocyte growth factor expressed by baculovirus. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 591−598.
(25) Yee, C. J.; DeFrances, M. C.; Bell, A.; Bowen, W.; Petersen, B.; Michalopoulos, G. K.; Zarnegar, R. Expression and characterization of biologically active human hepatocyte growth factor (HGF) by insect cells infected with HGF-recombinant baculovirus. *Biochemistry* **1993**, *32*, 7922−7931.
(26) Naldini, L.; Tamagnone, L.; Vigna, E.; Sachs, M.; Hartmann, G.; Birchmeier, W.; Daikuhara, Y.; Tsubouchi, H.; Blasi, F.; Comoglio, P. M. Extacellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. *EMBO J.* **1992**, *11*, 4825−4833.
(27) O'Reilly, D. R.; Miller, L. K.; Luckow, V. A. *Baculovirus Expression Vectors: A Laboratory Manual*; W. H. Freeman and Company: New York, 1992.
(28) Ramabhadran, T. V.; Gandy, S. E.; Ghiso, J.; Czernik, A. J.; Ferris, D.; Bhasin R.; Goldgaber, D.; Frangione, B.; Greengard, P. Proteolytic processing of human amyloid β protein precursor in insect cells. *J. Bio. Chem.* **1993**, *268*, 2009−2012.
(29) Pyle, L. E.; Barton, P.; Fujiwara, Y.; Mitchell, A.; Fidge, N. Secretion of biologically active human proapolipoprotein A−I in a baculovirus-insect cell system: protection form degradation by protease inhibitors. *J. Lipid Res.* **1995**, *36*, 2355−2361.
(30) Slack, J. M.; Kuzio, J.; Faulkner, P. Characterization of *v*-cath, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol.* **1995**, *76*, 1091−1098.
(31) Grosch, H.; Hasilik, A. Protection of proteolysis-prone recombinant proteins in baculovirus expression systems. *BioTechniques* **1998**, *24*, 930−934.
(32) Naggie, S.; Bentley, W. E. Appearance of protease activities coincides with p10 and polyhedrin-driven protein production in the baculovirus expression system: effects on yield. *Biotechnol. Prog.* **1998**, *14*, 227−232.
(33) Jiang, B.; Barniak, V.; Smith, R. P.; Sharma, R.; Corsaro, B.; Hu, B.; Madore, H. P. Synthesis of rotavirus-like particles in insect cells: comparative and quantitative analysis. *Biotechnol. Bioeng.* **1998**, *60*, 369−374.
(34) Shih, C. J.; Lin, R. W.; Wang, C. H. Establishment of a cell from *Spodoptera litura* (Lepidoptera: Noctuidae) and replication of *S. litura* nuclear polyhedrosis virus in vitro. *J. Invertebr. Pathol.* **1997**, *69*, 1−6.
(35) Wang, M. Y.; Vakharia, V.; Bentley, W. E. Synthesis of epoxide hydroxylase: a focus on infected cell. *Biotechnol. Bioeng.* **1993**, *42*, 240−247.
(36) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: New York, 1989.
(37) Lee, H. S.; Huang, G. T.; Sheu, J. C.; Chiou, L. L.; Horng, M. C.; Lai, M. Y.; Chen, D. S.; Lee, S. C. Lack of critical domains in the β-chain of hepatocyte growth factor. *Biochem. Biophys. Res. Commun.* **1995**, *210*, 1017−1024.
(38) Vernet, T.; Tessier, D. C.; Richardson, C.; Lalibrte, F.; Khouri, H. E.; Bell, A. W.; Storer, A. C.; Thomas, D. Y. Secretion of functional papain precursor from insect cells. *J. Biol. Chem.* **1990**, *265*, 16661−16666.
(39) Wang, M. Y.; Pulliam, T. R.; Valle, M.; Vakharia, V. N.; Bentley, W. E. Kinetic analysis of alkaline protease activity, recombinant protein production and metabolites for infected insect (Sf9) cells under different DO levels. *J. Biotechnol.* **1996**, *46*, 243−254.

Accepted December 7, 1999.

BP990143T