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**Generation of Engineered Recombinant Hepatocyte Growth Factor
Cleaved and Activated by Genenase I**

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Abstract

Hepatocyte growth factor (HGF) is biosynthesized as a biologically inactive, single-chain form (pro-HGF). Its activation is associated with cleavage at Arg494-Val495 into a two-chain mature form composed of disulfide-linked α - and β -chains. Because serum is a major source of HGF activator (the predominant serine protease responsible for the processing of pro-HGF), serum-free production of recombinant, two-chain HGF had not been established. In this study, to enable serum-free production of two-chain HGF, we generated engineered human pro-HGFs that can be specifically cleaved and activated by Genenase I. Since Genenase I specifically cleaves the C-terminus of the His-Tyr sequence, which does not exist in human HGF, Arg494 (the C-terminus of the HGF α -chain) was replaced by His-Tyr, Ala-Ala-His-Tyr, Pro-Gly-His-Tyr, or Pro-Gly-Ala-Ala-His-Tyr. Genenase I cleaved engineered pro-HGFs specifically at the replaced amino acid sequences, forming a disulfide-linked two-chain form. The cleavage was most efficient in the case of the Pro-Gly-Ala-Ala-His-Tyr sequence, and cleaved HGFs displayed biological activities identical to those of wild-type HGF. Considering a potential medical application of HGF, the present technique is valuable because it enables the production of recombinant, two-chain HGF entirely without serum and extends the choice of host cells and organisms for recombinant production.

Key words: hepatocyte growth factor, activation, Genenase I, serum

1. Introduction

Hepatocyte growth factor (HGF), originally identified as a potent mitogenic protein for hepatocytes (Nakamura et al., 1984), has multiple biological activities on a variety of cells through activation of the Met receptor tyrosine kinase. HGF-Met signaling mediates a diverse array of biological and physiological processes, including the development and morphogenesis of organs, epithelial-mesenchymal interactions and the regeneration of tissues (Matsumoto and Nakamura, 1997; Birchmeier and Gherardi, 1998). Changes in the gene expression of HGF following tissue injuries, together with the retarded repair and augmented damage of tissues caused by the neutralization of HGF (Mizuno and Nakamura, 2005) or by the loss-of-function mutation in the Met (Borowiak et al., 2004; Huh et al., 2004), indicate that HGF plays a trophic role in the physiological repair of various tissues, including but not limited to the liver. The supplemental administration of recombinant HGF in experimental animals displays remarkable therapeutic effects on various types of injuries and disease models in different tissues (Matsumoto and Nakamura, 2001; Funakoshi and Nakamura 2003; Mizuno and Nakamura, 2007).

HGF is a disulfide-linked heterodimeric molecule composed of a 69 kD α -chain and a 34 kD β -chain (Nakamura et al., 1989; Miyazawa et al., 1989). The α -chain contains the N-terminal hairpin domain and four kringle domains, and the β -chain contains a serine protease-like domain. It should be emphasized that HGF is biosynthesized and secreted in a single-chain precursor form (pro-HGF), which is biologically inactive, and that extracellular processing into a two-chain form is associated with conversion from biologically inactive to active HGF (Mizuno et al., 1992; Naka et al., 1992; Gak et al., 1992). Specific extracellular cleavage occurs between Arg494 (C-terminus of the α -chain) and Val495 (N-terminus of the β -chain) of HGF, and several serine proteases responsible for this cleavage have been reported, including HGF activator (HGFA) (Miyazawa et al., 1993), matriptase (Lee et al., 2000), hepsin (Kirchhofer et al., 2005), urokinase-type plasminogen activator (Naldini et al., 1995), and tissue-type plasminogen activator (Mars et al., 1993).

Among these serine proteases, HGFA is considered the most relevant activator of single-chain HGF both kinetically and physiologically. In HGFA-deficient mice, HGF-processing activity was absent in the sera, and impaired regeneration was seen in an intestinal injury model (Itoh et al., 2004). Like HGF, HGFA is biosynthesized as an

enzymatically inactive, single-chain form and secreted into the plasma as this inactive form (Miyazawa et al., 1993). In response to tissue injury, the single-chain pro-HGFA is cleaved and activated by thrombin under the blood coagulation cascade (Shimomura et al., 1993). Consistent with this, in cultures of host cells for expression of recombinant HGF, only biologically inactive, single-chain HGF (but not biologically active two-chain HGF) is expressed without supplementation of sera, such as fetal bovine serum (FBS) (Mizuno et al., 1992; Shimomura et al., 1992; Miyazawa et al., 1993). Therefore, the active form of recombinant HGF has been produced by serum-supplemented culture systems. However, in production of recombinant HGF for medical use, serum-free systems are preferred because serum use requires careful selection and validation of serum sources to avoid contamination by viruses or prions.

This study was performed to enable production of recombinant HGF in an active form under serum-free conditions. To achieve this, we designed engineered human pro-HGFs that contain specific amino acid sequences at the cleavage site between the α - and β -chains of HGF so that single-chain pro-HGFs can be cleaved by a specific protease. We selected Genenase I (Carter et al., 1989; Carter et al., 1991), a H64A mutant of subtilisin, as the specific protease for four reasons: 1) the minimum amino acid sequence recognized by Genenase I is only His-Tyr or Tyr-His; 2) neither His-Tyr nor Tyr-His are found in the human HGF sequence; 3) Genenase I can be produced in an active form without serum; and 4) the enzyme reaction can be achieved under mild conditions (e.g., does not require reducing conditions, which inactivate HGF).

In this study, we demonstrate that engineered pro-HGFs can be specifically cleaved by Genenase I at the junction site and that the cleaved HGF show typical biological activities. We propose that engineered pro-HGF cleavable by Genenase I can be applied to the production of biologically active, two-chain HGF under serum-free conditions in different types of host cells and organisms for recombinant production.

2. Materials and methods

2.1 Design of engineered HGFs

We designed four types of engineered pro-HGFs (Fig. 1). In wild-type human HGF,

cleavage for activation occurs between Arg494 and Val495. The amino acid residues of the C-terminal region (~Arg494) of the α -chain were changed, while the N-terminus of the β -chain was not changed, because the N-terminus of the β -chain plays a critical role in the functional interaction of the HGF β -chain with the Met receptor (Kirchhofer et al., 2007). The newly introduced sequences at the C-terminal region of the α -chain were HY (GC-1), AAHY (GC-2), PGHY (GC-3) and PGAAHY (GC-4). According to information from the provider of Genenase I (New England Biolabs, Beverly, MA, USA), the PGAAHY sequence is most efficiently recognized by Genenase I. The AAHY and PGHY sequences were designed by modification of the PGAAHY sequence. Because Genenase I cleaves the C-terminal side of the Tyr residue of the His-Tyr sequence, the engineered precursors were expected to be cleaved by Genenase I at the C-terminal of Tyr494.

2.2 Generation of recombinant baculoviruses for expression in Sf9 cells

To exclude the potential influence of sugar chains on the biochemical characteristics of the engineered HGF produced by different host species in future applications, nonglycosylated HGF (Fukuta et al., 2005) was used as a wild-type HGF. The nonglycosylated HGF was cleaved and activated in the presence of FBS, and it was indistinguishable from glycosylated-native HGF in both biochemical and biological characteristics and activity (Fukuta et al., 2005).

The wild-type cDNA encoding human nonglycosylated HGF (5 amino acids-deleted type) was cloned into the BamH I/Xba I site of the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA), and the vector was designated pcDNA3.1(+)-HGF-NG. By using this wild-type cDNA as a template, cDNAs for engineered HGFs were generated by site-directed mutagenesis. Mutagenesis was performed so that the C-terminal region of the α -chain of HGF was replaced as follows: L493H-R494Y (GC-1), K491A-Q492A-L493H-R494Y (GC-2), K491P-Q492G-L493H-R494Y (GC-3), and K489P-T490G-K491A-Q492A-L493H-R494Y (GC-4). (The numbering of amino acids is indicated on the basis of the amino acid sequence of full-length HGF.) Mutagenic PCR was performed so that mutagenic primers were incorporated into the sequence between the BamH I and Xba I sites of the wild-type vector. The mutagenic primers used were: 5'-(p)CCAAAACGAAACAACACTATGTTGTAAATGGGATTCCAAC-3' (for GC-1);

5'-(p)GCCAAAACGGCCGCACACTATGTTGTAAATG-3' (for GC-2);
 5'-(p)CCAAAACGCCAGGACACTATGTTG-3' (for GC-3); and
 5'-(p)GTAATATCTTGTGCCCCAGGGGCCGCACACTATGTTGTAAATGG-3' (for GC-4). The BamH I/Xba I fragment of the mutants was cloned into the BamH I/Xba I site of the pcDNA3.1(+) vector. Constructions were confirmed by DNA sequencing.

Recombinant baculoviruses for the expression of the engineered pro-HGFs were generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen), which utilizes a baculovirus shuttle vector (bacmid) propagated in *Escherichia coli*. The above-described BamH I/Xba I fragments cloned into the pcDNA3.1(+) vector were transferred to the BamH I/Xba I site of the pFastBac1 vector contained in the Bac-to-Bac system, where the recombinant gene is controlled by the *Autographa californica* multiple nuclear polyhedrosis virus polyhedron promoter, and the expression cassette forms a mini T7 transposon.

The pFastBac1 vectors were transformed into DH10Bac *E. coli* competent cells (Invitrogen) that contain a bacmid and a helper plasmid encoding transposase. In the DH10Bac cells, the transposition between the expression cassette contained in the pFastBac1 vector and the bacmid occurs with the aid of the helper plasmid, and the recombinant bacmids are generated. The recombinant bacmids were isolated and transfected into insect *Spodoptera frugiperda* Sf9 cells to generate recombinant baculoviruses. The recombinant baculoviruses were amplified, tittered and used for the expression of engineered proteins.

2.3 Expression and purification of recombinant proteins

Wild-type pro-HGF was expressed in human embryonic kidney cell line 293T (HEK293T) cells (American Type Culture Collection CRL-11268). The HEK293T cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (obtained from Equitech-Bio, Inc., Kerrville, TX, USA). Before transfection, the medium was changed to serum-free DMEM, and the pcDNA3.1(+)-HGF-NG vector was introduced into 90–95% confluent HEK293T cells, using Lipofectamine 2000 transfection reagent (Invitrogen). After the cells were cultured for 6 h, the medium was changed to fresh serum-free DMEM containing 1 µg/ml heparin, and the cells were cultured for 3 days.

Engineered pro-HGFs were expressed in Sf9 cells (obtained from Novagen, Madison, WI, USA). Sf9 cells were first cultured in T-flasks with Sf900II medium (Invitrogen)

containing 10% FBS at 27°C. Then the cells were adapted to serum-free Sf900II medium and cultured in Spinner flasks. The suspension of Sf9 cells was infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 10 and cultured for 3 days.

The culture supernatants of wild-type pro-HGF or engineered pro-HGFs were loaded onto a Hi-Trap heparin HP column (GE Healthcare, Buckinghamshire, UK) equilibrated with 50 mM Tris/HCl buffer (pH 7.5) containing 0.3 M NaCl and 0.01% Tween 80. After washing with 50 mM Tris/HCl buffer (pH 7.5) containing 0.7 M NaCl and 0.01% Tween 80, adsorbed proteins were eluted with a linear gradient of 0.7-2.0 M NaCl in 50 mM Tris/HCl buffer (pH 7.5) containing 0.01% Tween 80. HGF in each fraction was determined using an enzyme-linked immunosorbent assay (ELISA). Fractions containing HGF were applied onto a HiTrap SP HP column (GE Healthcare) equilibrated with 50 mM Tris/HCl buffer (pH 7.5) containing 0.3 M NaCl and 0.01% Tween 80. After washing with the same buffer, proteins were eluted with a linear gradient of 0.3-1.0 M NaCl in 50 mM Tris/HCl buffer (pH 7.5) containing 0.01% Tween 80. The purified samples were passed through a 0.22 µm filter membrane, and the concentrations of proteins were determined using a BCA Protein Assay kit (PIERCE, Rockford, IL, USA) with bovine serum albumin as a standard. The purity of the engineered pro-HGFs was more than 98% as determined by SDS-PAGE and protein staining with Coomassie Brilliant Blue (CBB) R-250.

2.4 Treatment of pro-HGFs with Genenase I

Five µg of recombinant pro-HGFs were treated with Genenase I (New England Biolabs, Beverly, MA, USA) by changing the ratio of Genanase I to the pro-HGFs. The reaction mixture, which contained 50 mM Tris/HCl (pH 7.5), 1 M NaCl and 0.01% Tween 80, was incubated at 22°C for 12 h.

2.5 SDS-PAGE and western blotting

SDS-PAGE was performed using a 4-20% gradient gel. Proteins were stained with CBB R-250. For western blotting, the gels were electroblotted onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) and probed with a biotinylated polyclonal antibody against human HGF. Immunoreactive proteins were detected with horseradish-peroxidase-conjugated streptavidin (GE Healthcare) and ECL (enhanced chemiluminescence) reagents (GE Healthcare).

2.6 N-terminal amino acid analysis of the β -chain of HGF

After treatment of GC-4 with Genenase I, the sample was subjected to SDS-PAGE under a reducing condition. Separated subunits were transferred onto a PVDF membrane and stained by CBB. The band of the β -chain was excised, and the N-terminal amino acid sequence was analyzed by automated Edman degradation using a Model 477A Protein Sequencer (Applied Biosystems, Foster City, CA, USA) equipped with a Model 120A PTH Analyzer (Applied Biosystems).

2.7 Assay of biological activities

Cell-scattering assays using MDCK (Madin-Darby canine kidney) (clone 3B) renal epithelial cells were performed as described in a previous study (Fukuta et al., 2005). MDCK cells were a gift from Dr. Roberto Montesano (Department of Morphology, University of Geneva Medical School, Switzerland). The mitogenic activity of the HGF was assayed by measuring the proliferation of Mv1Lu mink lung epithelial cells (RCB0996, obtained from RIKEN BioResource Center, Japan), according to the method described previously (Rafferty et al., 2001) with some modification. The cells were seeded on a 96-well plate at 10,000 cells per well in a RPMI 1640 medium supplemented with 2% FBS and 250 pg/ml TGF- β 1. The HGF samples were added; the cells were cultured for 48 h; and a proliferation of the cells was assessed by a colorimetric method using a Cell Titer96 Aqueous Cell Proliferation Assay kit (Promega, Madison, WI, USA). The mitogenic activity of the HGF was also measured using adult rat hepatocytes in a primary culture, as described previously (Fukuta et al., 2005). In every assay, Genenase I-treated samples were added to the cells without eliminating the Genenase I, because the addition of only Genenase I, in the same concentration as in the Genenase I-treated samples, had not induced cell response.

3. Results and discussion

3.1 Treatment of pro-HGFS with Genenase I

The primary structures of the processing sites of wild-type human HGF and four engineered human HGFs (GC-1, -2, -3 and -4) are shown in Fig. 1. Wild-type pro-HGF was

expressed in HEK293T cells in a serum-free culture and was partially purified by heparin affinity chromatography. Engineered pro-HGFs were expressed by the baculovirus-Sf9 system under serum-free culture conditions, and these pro-HGFs were purified from the culture supernatants by heparin-affinity and cation-exchange chromatography.

Based on potential substrate specificity, Genenase I was expected to not cleave wild-type pro-HGF at any site and to cleave engineered pro-HGFs only at the introduced sequence. We first examined whether or not Genenase I cleaves wild-type pro-HGF. The partially purified pro-HGF was treated with either Genenase I or FBS, and the resultant samples were analyzed by SDS-PAGE, followed by western blotting using an anti-human HGF polyclonal antibody (Fig. 2). The wild-type pro-HGF was detected as a single band at a position of 80 kDa under a reducing condition (lane 1). When the wild-type pro-HGF was treated with FBS, the single-chain HGF was split into the α -chain (53 kDa) and the β -chain (26 kDa) (lane 3). Thus, wild-type pro-HGF was cleaved into the α - and β -chains by the serum. Under a non-reducing condition, the serum-treated HGF (lane 6) was detected as a single band at the same position as the non-treated pro-HGF (lane 4), indicating that the α - and β -chains were linked by an S-S bond. On the other hand, the band of pro-HGF, after treatment with Genenase I, was detected as a single band at the same position as the sample before the treatment, under both reducing and non-reducing conditions (lanes 2 and 5), indicating that wild-type pro-HGF was not cleaved by Genenase I.

Next, engineered pro-HGFs were treated with Genenase I (22°C, 12 h) by changing the ratio of Genenase I to pro-HGF from 0.0001:1 to 0.1:1 (wt:wt), respectively. The SDS-PAGE of Genenase I-treated samples, under either reducing or non-reducing conditions, indicated that all engineered pro-HGFs were cleaved by Genenase I into two subunits corresponding to the α - and β -chains of HGF (Fig. 3A). Because Genenase I did not cleave wild-type HGF, engineered pro-HGFs were considered to be cleaved at the introduced sequences. However, the cleavage efficiency varied depending on the introduced sequence. In GC-1 (C-terminus of the α -chain is HY), cleavage by Genenase I was not seen when the ratio of Genenase I:GC-1 was less than 0.1:1. When the ratio of Genenase I:GC-1 was 0.1:1, the GC-1 was significantly cleaved, whereas uncleaved GC-1 remained at a significant level. On the other hand, GC-2, -3 and -4 were efficiently cleaved by Genenase I. In the case of GC-2 (C-terminus of the α -chain is AAHY) and GC-3 (C-terminus of the α -chain is PGHY),

significant specific cleavage was seen at the ratio of 0.001:1 (Genenase I:pro-HGF), and almost complete cleavage was seen at the ratio of 0.01:1 and 0.1:1. In GC-4 (C-terminus of the α -chain is PGAAHY), significant cleavage was seen at the ratio of 0.0001:1 (Genenase I:GC-4), and almost complete cleavage was seen at the ratio of 0.01:1 and 0.1:1. In SDS-PAGE under non-reducing conditions, engineered HGFs, both untreated and treated with Genenase I, were detected as a single band at the same position as wild-type, two-chain HGF (Fig. 3B). Therefore, the α - and β -chains of Genenase I-treated HGFs were considered to be linked by an S-S bond, as with the case of an active form of wild-type HGF.

To confirm the cleavage site of engineered HGF by Genenase I, we analyzed the N-terminal amino acid sequence of the β -chain of Genenase I-treated GC-4. The N-terminal sequence was determined to be VVNGI. This sequence was identical to that of the wild-type HGF β -chain (Fig. 1). Thus, Genenase I specifically cleaved GC-4 between Tyr494 and Val495, as expected.

3.2 Time course of cleavage of GC-4 by Genenase I

Because GC-4 was most efficiently cleaved by Genenase I, we analyzed the time course of GC-4 cleavage in order to optimize the reaction condition (Fig. 4). Genenase I was added at a ratio of 0.01:1 or 0.001:1 (Genenase I:GC-4), and the reaction was performed at 22°C. The extent of cleavage was chased for 12 h (for 0.01:1) or 24 h (for 0.001:1). When the ratio was 0.001:1, the cleavage of GC-4 began at 30 min and was mostly completed within 12 h. Excess cleavage was not observed even when the reaction continued for 24 h. When the ratio was 0.01:1, the cleavage was mostly complete at 1 h, and excess cleavage was not observed even when the reaction was continued for 12 h. Thus, the cleavage reaction of GC-4 with Genenase I was practical at a ratio of 0.001:1 (Genenase I: GC-4) at 22°C for 12 to 24 h.

3.3 Biological activities of Genenase I-treated pro-HGFs

We next examined if the engineered HGFs converted to the two-chain form by the Genenase I display the biological activities of HGF. Cleaved HGFs were prepared by treating the pro-HGFs with Genenase I at a weight ratio (Genenase I:pro-HGF) of 0.1:1 (GC-1), 0.01:1 (GC-2), 0.01:1 (GC-3) and 0.001:1 (GC-4) at 22°C for 12 h. In a monolayer culture of MDCK renal epithelial cells, untreated engineered HGFs did not stimulate a

scattering of cells, whereas the Genenase I-treated HGFs stimulated cell scattering in a dose-dependent manner (Fig. 5). The activities of GC-2, -3 and -4 were almost comparable to that of wild-type HGF, while the activity of GC-1 was somewhat weak compared to others. The weak activity of GC-1 is probably due to insufficient cleavage of GC-1 by Genenase I, as described above.

In a mitogenic assay to measure the proliferation of Mv1Lu cells, GC-4 without Genenase I treatment did not stimulate cell proliferation (Fig. 6A). Untreated GC-1, GC-2 and GC-3 also had no effect on cell proliferation (not shown). In contrast, Genenase I-treated HGFs stimulated the proliferation of Mv1Lu cells. The activities of GC-2, GC-3 and GC-4 were almost comparable to that of wild-type HGF. The activity of GC-1 was somewhat weak at concentrations of less than 30 ng/ml, possibly due to insufficient cleavage. The mitogenic activity of GC-4 was also assessed by measuring the DNA synthesis in rat hepatocytes in a primary culture (Fig. 6B). The GC-4 treated with Genenase I stimulated DNA synthesis in the rat hepatocytes in a dose-dependent manner, and the activity was almost comparable to that of the active form of wild-type HGF. Thus, the engineered HGFs converted to the two-chain form by Genenase I had biological activity comparable to that of wild-type HGF.

3.4 Discussion

We demonstrated that engineered single-chain pro-HGF was cleaved into two-chain HGF by Genenase I specifically at the junction of the α - and β -chains of HGF. This was coupled with a conversion from biologically inactive to active HGF. Since recombinant Genenase I can be produced as an active enzyme using bacteria such as *Bacillus subtilis* (Carter et al., 1989), our finding provides a novel technique for the preparation of biologically active, two-chain HGF completely under serum-free conditions. One potential concern with medical application of the present technique is the antigenicity of engineered HGF due to the substitution of amino acids at the junction site. Although the number of substituted amino acids for cleavage by Genenase I is small (four or six amino acids for effective cleavage), the potential antigenicity should be evaluated carefully. On the other hand, the production of wild-type, two-chain HGF with the use of serum requires the assurance of serum quality to avoid contamination by viruses or prions. The advantage of our method is that the production of recombinant, two-chain HGF is possible without serum

quality control. Furthermore, this method is applicable to host cells and organisms other than insect or mammalian cells (e.g., *E. coli*, yeasts, and plants), which do not require serum for culture or growth. The administration of recombinant HGF has remarkable therapeutic effects on various disease models in different tissues (Matsumoto and Nakamura 2001; Funakoshi and Nakamura 2003; Mizuno and Nakamura 2007). We predict that this technique will be beneficial for future industrial production of recombinant HGF for medical use.

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Abbreviations

HGF, hepatocyte growth factor; HGFA, hepatocyte growth factor activator; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MDCK, Madin-Darby canine kidney.

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Figure legends

Fig. 1. Schematic structure of HGF and partial amino acid sequences of wild type and engineered HGFs.

In wild-type human HGF, specific cleavage into two-chain HGF occurs between Arg494 and Val495 (arrow). Four engineered human HGFs were designed so that the C-terminal region of α -chain is substituted by Genenase I-cleavable sequences.

Fig. 2. Lack of activity of Genenase I to cleave wild-type pro-HGF

Partially purified wild-type pro-HGF (200 ng) was treated with FBS (37°C, 12 h) or Genenase I (22°C, 12 h). The treated samples were subjected to SDS-PAGE under reducing or non-reducing conditions and analyzed by western blotting using an anti-human HGF polyclonal antibody.

Fig. 3. Cleavage of engineered HGFs by Genenase I

Engineered pro-HGFs were treated with Genenase I at 22°C for 12 h by changing the ratio of Genenase I to pro-HGFs. The samples were subjected to SDS-PAGE under reducing (A) or nonreducing (B) conditions, and proteins were stained with CBB. In panel A, engineered pro-HGFs were untreated (indicated as “-”) or treated with Genenase I. The ratio of Genenase I: pro-HGF was changed at 0.0001:1, 0.001:1, 0.01:1, and 0.1:1 (wt:wt). In samples treated with Genenase I at 0.1:1, the band of Genenase I is seen. The profile of Genenase I alone under SDS-PAGE is indicated in the rightmost lane. In panel B, engineered HGFs were respectively treated with Genenase I at the ratio (Genenase I:pro-HGF) of 0.1:1 (GC-1), 0.01:1 (GC-2), 0.01:1 (GC-3), 0.001:1 (GC-4) at 22°C for 12 h. The band of Genenase I is observed only in GC-1 due to the high amount of Genenase I used.

Fig. 4. Time course of cleavage of GC-4 by Genenase I

Genenase I was added at a ratio of 0.01:1 or 0.001:1 (Genenase I:GC-4), and the reaction was carried out at 22°C for 12 or 24 h. The samples were analyzed by SDS-PAGE under a reducing condition.

Fig. 5. Cell scattering activity of Genenase I-treated HGFs

Engineered pro-HGFs, untreated or treated with Genenase I, were added to a culture of MDCK cells. The MDCK cells were cultured for 18 h and stained with crystal violet.

Fig. 6. Mitogenic activity of Genenase I-treated HGFs on Mv1Lu cells (A) and rat hepatocytes in primary culture (B)

Mv1Lu cells were cultured in the absence or presence of HGFs for 48 h, and the proliferation of the cells was determined by a colorimetric assay. Hepatocytes were cultured in the absence or presence of HGFs for 20 h, and DNA synthesis was determined by pulse-labeling with [³H]thymidine for 6 h.