Expression and Characterization of Biologically Active Human Hepatocyte Growth Factor (HGF) by Insect Cells Infected with HGF-Recombinant Baculovirus†

Cindy J. Yee, Marie C. DeFrances, Aaron Bell, William Bowen, Bryon Petersen, George K. Michalopoulos, and Reza Zarnegar*

Department of Pathology, Division of Cellular and Molecular Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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ABSTRACT: A cDNA containing the entire coding sequence of human hepatocyte growth factor (HGF) [also known as scatter factor (SF)] was inserted into the genome of *Autographa california* nuclear polyhedrosis virus (baculovirus) adjacent to the polyhedrin promoter by homologous recombination. Insect cells (*Spodoptera frugiperda*) infected with the recombinant virus secrete relatively high levels (3–8 mg/L) of biologically active HGF into the culture medium. The recombinant HGF induces pronounced morphological changes and scattering of primary cultures of rat, mouse, and human hepatocytes within 24 h after plating and stimulates DNA synthesis in these cells with the same magnitude as native HGF derived from human placenta or rabbit serum. The human recombinant HGF produced by the insect cells is N-glycosylated, binds to heparin like native HGF, and is recognized by polyclonal antiserums raised against human or rabbit HGF as assessed by immunoblot, ELISA, and immunoneutralization experiments. Metabolic radiolabeling with L-[^{35}S]methionine (pulse-chase experiments) as well as Western blot analysis indicates that the recombinant HGF is synthesized and secreted by the infected insect cells as the unprocessed single-chain form (pro-HGF) when the cells are cultured in serum-free medium. However, when the infected insect cells are cultured in insect culture medium (Grace's medium) containing fetal bovine serum, the secreted HGF is present mainly in the mature heterodimeric form. Addition of serum to the baculovirus-expressed single-chain [^{125}I]HGF in a cell-free system results in conversion to the heterodimeric two-chain form, and the activation is prevented by the serine protease inhibitor PMSF. Incubation of ^{125}I-labeled pro-HGF with rat liver or spleen extracts resulted in conversion of pro-HGF to the heterodimeric two-chain form. A truncated form of HGF containing the N-terminal portion of HGF (kringles 1–3) was also produced in the same expression system. This deleted HGF, by itself, did not have any detectable biological activity; however, it abrogated the stimulatory effects of full-length HGF on hepatocytes. This is the first successful production of bioactive recombinant HGF in large quantities, which will allow purification on the milligram scale of pro-HGF and will permit future studies to elucidate pathways involved in HGF activation by its target tissues.

Hepatocyte growth factor (HGF)[1] is a multifunctional heparin-binding polypeptide that was originally identified on the basis of its ability to stimulate DNA synthesis in primary cultures of rat hepatocytes (Nakamura et al., 1987; Gohda et al., 1988; Zarnegar & Michalopoulos, 1989; Zarnegar et al., 1989). Subsequent investigations revealed that HGF is also a potent mitogen for many other cell types of epithelial origin such as kidney tubular epithelial cells, keratinocytes, endothelial cells, and melanocytes (Kan et al., 1991; Matsumoto et al., 1991a,b; Rubin et al., 1991). In addition to its mitogenic effects, HGF also stimulates epithelial cell motility and dispersion (it was independently identified and characterized as scatter factor or SF) (Gherardi & Stoker, 1990; Naldini et al., 1991; Weidner et al., 1991), and formation of tubular-like structures upon addition to MDCK cells in tissue culture (Montesano et al., 1991). Other activities that have been described for HGF include its cytotoxic and cytostatic properties toward several tumor cell lines (Higashio et al., 1990; Tajima et al., 1991). HGF is expressed at low levels during embryonic development and is present in various tissues of adult and embryonic rat, human, and presumably other vertebrates (Zarnegar et al., 1990; Wolf et al., 1991; DeFrances et al., 1992).

HGF purified from rabbit serum, rat platelets, or human plasma is a heterodimeric glycoprotein consisting of a heavy (α chain) and a light chain (β chain) with M_r's of 58 000–69 000 and 30 000–34 000, respectively (Nakamura et al., 1987; Gohda et al., 1988; Zarnegar & Michalopoulos, 1989; Zarnegar et al., 1989). On the other hand, HGF purified from conditioned medium of a human embryonic lung fibroblast cell line or human placenta exists predominantly as an unprocessed single-chain pro-HGF polypeptide with an M_r of 87 000–92 000 (Weidner et al., 1990; Rubin et al., 1991; Hernandez et al., 1992). The predicted structure of human HGF (based on its cDNA nucleotide sequence) indicates that the two polypeptide chains of mature heterodimeric HGF are transcribed from a single open reading frame to yield the pre-pro-HGF molecule coding for 728 amino acids (Miyazawa et al., 1989; Nakamura et al., 1989). The signal peptide of

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* To whom correspondence should be addressed.

[1] Abbreviations: AcNPV, *Autographa california* nuclear polyhedrosis virus; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FPLC, fast performance liquid chromatography; High 5, *Trichoplusia ni 5B1-4* insect cells; HGF, hepatocyte growth factor; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; RT–PCR, reverse transcriptase–polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SF, scatter factor; Sf9, *Spodoptera frugiperda* insect cells; t-PA, human tissue-type plasminogen activator; u-PA, human urokinase-type plasminogen activator.

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31 amino acids at the amino terminus of pre-pro-HGF is removed to yield the pro-HGF precursor (Nakamura et al., 1989). Structurally, HGF is similar to plasminogen (presence of kringle domains in the α chain and consensus sequence for serine proteases in the β chain with overall 39% amino acid sequence homology), but it does not possess proteolytic activity (Nakamura et al., 1989). It has been proposed that pro-HGF is converted to the mature heterodimeric form by a serine protease-like enzyme such as tissue-type plasminogen activator/urokinase-type plasminogen activator proteases or plasmin (Nakamura et al., 1989; Rubin et al., 1991). Such an enzyme should cleave pro-HGF at Arg^{494}–Val^{495} to generate the α and the β chains of mature HGF. These two chains are predicted to be composed of 464 and 234 amino acids with calculated M_r's of 50,808, and 26 089, respectively (Nakamura et al., 1989).

In vitro mutagenesis studies have shown that although pro-HGF binds to the HGF receptor (the product of the protooncogene c-MET) with nearly the same affinity as the mature heterodimeric form, it is not biologically active, and it appears that protease conversion to the heterodimeric form is required for bioactivity of HGF (Lokker et al., 1992). At the present time, the exact *in vivo* mode of HGF activation is not well understood. The physiological role of HGF *in vivo* is also unclear, and studies of the effects of administering exogenous HGF to intact animals have been hampered by a lack of large quantities of HGF. Attempts to express recombinant HGF have been limited to transient or stable expression of HGF cDNA in COS cells with small yields (Miyazawa et al., 1989; Nakamura et al., 1989). Other sources of HGF in the past have been rat platelets (more than 3000 rats were used to isolate 60 μg of purified HGF for amino acid sequencing analysis) (Nakamura et al., 1989), rabbit serum and human plasma (more than 100 L to purify 50–100 μg of HGF) (Zarnegar & Michalopoulos, 1989), human placenta (more than 50 placentas to obtain 1 mg of HGF) (Hernandez et al., 1992), or conditioned medium from the MRC-5 human lung embryonic fibroblast cell line (more than 100 L to obtain 50 μg of purified HGF) (Rubin et al., 1991). As can be concluded from these findings, HGF purification from these sources is costly, time consuming, and laborious. Moreover, HGF prepared from these sources has been shown to be comprised of a mixture of single-chain pro-HGF as well as the mature heterodimeric form, making these preparations undesirable for certain studies such as the mode of HGF activation by proteases.

In the present paper, we report the production of relatively large quantities (3–8 mg/L) of bioactive human recombinant HGF produced by a baculovirus-based expression system and describe some of its biochemical and biological properties.

MATERIALS AND METHODS

Construction of a Human Recombinant HGF Baculovirus Plasmid Vector. A full-length human HGF cDNA (*Bam*HI/*Dra*I fragment) was isolated from a human placental cDNA library and subcloned into the *Bam*HI site of the plasmid pSPORT (GIBCO BRL, Grand Island, NY) as we described previously (Zarnegar et al., 1991). This clone encompasses nucleotides −26 to +2262 of the human placental HGF cDNA, described by Miyazawa et al. (1989); however, it differs from that clone since it contains a five amino acid deletion in the first kringle region (amino acids 163–167, PheLeuProSerSer). This variant of the HGF cDNA has also been isolated from cDNA libraries prepared from a human lung fibroblast cell line (Rubin et al., 1991) and human leukocytes (Seki et al., 1990) and is believed to arise from alternative RNA splicing. To clone this HGF cDNA into the *Nhe*I site of the baculovirus transfer vector pBlueBac (Invitrogen, San Diego, CA; see Figure 1), the protruding ends of the HGF cDNA *Bam*HI fragment were filled with DNA polymerase (Klenow fragment, GIBCO BRL). The resulting blunt-ended insert was ligated to phosphorylated *Nhe*I linkers (Pharmacia LKB, Piscataway, NJ), digested with *Nhe*I, purified by agarose gel electrophoresis, and then ligated to pBlueBac that had been treated with *Nhe*I and BAP according to established procedures. The orientation of the human HGF cDNA insert in pBlueBac with regard to the polyhedrin promoter was determined by restriction enzyme analysis.

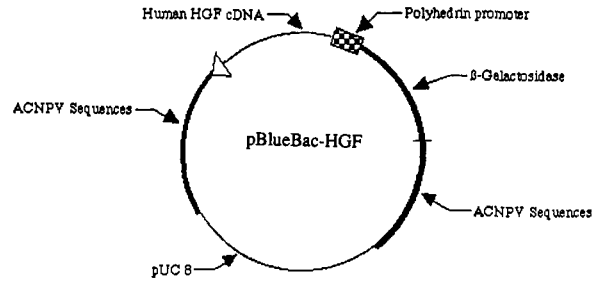

FIGURE 1: Construction of recombinant baculovirus transfer vector containing human HGF cDNA. A full-length human HGF cDNA was inserted into the *Nhe*I site of the pBlueBac transfer vector (Invitrogen) adjacent to the polyhedrin promoter as described under Materials and Methods. The inserted gene provides the translation initiation signal, resulting in a nonfused protein.

Construction of a Truncated HGF Baculovirus Plasmid Vector. To generate the deleted form of HGF, we digested the recombinant HGF baculovirus plasmid vector containing the full-length HGF cDNA with *Bgl*II which does not cut the vector, but cuts HGF cDNA at nucleotides 1204 and 1716. This deletion resulted in a truncated form containing the first 404 amino acids of the α chain which includes the hairpin loop, kringles 1–3, and 16 amino acids of kringle 4. The truncated HGF has no β chain due to the creation of a new stop codon at position 1775. From the mutated cDNA sequence, the truncated HGF protein is predicted to have an M_r of approximately 48 000.

Production of Recombinant Baculovirus. A baculovirus expression kit (MAXBAC, purchased from Invitrogen) was used to construct recombinant baculovirus containing the human HGF cDNA. Approximately 2.5×10^6 Sf9 (*Spodoptera frugiperda*) cells were cotransfected with 1 μg of wild-type AcNPV DNA and 2 μg of pBlueBac plasmid containing the HGF cDNA as recommended by the supplier (Invitrogen). Six days posttransfection, the culture supernatant was saved and used to isolate the recombinant baculovirus. Two rounds of plaque purification were performed to isolate the recombinant baculovirus (producing blue plaques) from the predominantly wild-type baculovirus (white plaques). The presence of the HGF cDNA insert and the expression of its mRNA in the recombinant virus were confirmed by PCR (polymerase chain reaction) and RT–PCR using HGF-specific primers as we described previously (DeFrances et al., 1992).

Production of Human HGF in S. frugiperda Cells. Logarithmically growing Sf9 or High 5 (*Trichoplusia ni 5B1-4*) cells at 1×10^6/mL were infected with recombinant baculovirus at a multiplicity of infection of 2:1. Infected cells were cultured in Grace's insect medium (Invitrogen) supplemented with 10% FBS or Ex-Cell 400 serum-free medium (JRH Biosciences, Lenexa, KS) containing 50 μg/mL gentamicin. Cells were cultured in either T-flasks (High 5 cells)

or spinner flasks and incubated at 27.5 °C. Aliquots of culture medium were removed from day 2 through day 7 postinfection and assayed for the presence of HGF by bioassay (stimulation of DNA synthesis in primary cultures of rat hepatocytes), ELISA, and immunoblot as we described previously (Nakamura et al., 1987; Weidner et al., 1990). Control culture supernatants from insect cells grown in the absence of any virus or infected with wild-type virus were included in these experiments.

Analysis of Proteins Expressed by Recombinant Virus. Insect cells infected with wild-type, HGF-recombinant baculovirus or uninfected cells were metabolically labeled for 4 h with L-[^{35}S]methionine (50 μCi/mL; Amersham, Arlington Heights, IL) in methionine-free Grace's medium beginning 2 days after infection and continuing on a daily basis until day 7. After labeling, the cell extracts and culture supernatants were analyzed by SDS–PAGE and autoradiography. In pulse-chase experiments 4 days after infection, which corresponds to the peak of HGF protein synthesis, insect cells were pulsed with L-[^{35}S]methionine for 15 min as above and chased in Grace's medium containing 90 μg/mL unlabeled methionine. At various times after the chase, culture supernatants and cell extracts were prepared for further analyses.

Purification of Recombinant HGF. HGF was purified from the culture supernatants of insect cells 7 days after infection with recombinant baculovirus by heparin-affinity chromatography as for native HGF (Zarnegar & Michalopoulos, 1989; Hernandez et al., 1992). Briefly, 100–500 mL of culture supernatant (Ex-Cell 400 serum-free medium or Grace's insect medium supplemented with 10% FBS) was adjusted to 0.4 M NaCl and applied to a heparin–agarose column (20-mL bed volume attached to a Pharmacia FPLC apparatus) that had been equilibrated in 0.4 M NaCl. The column was washed with 100 mL of 0.5 M NaCl and eluted with a linear gradient of NaCl (0.5–1.5 M NaCl). Fractions of 2 mL were collected, and 2-μL aliquots from each fraction were subjected to bioassay for HGF (stimulation of DNA synthesis in primary cultures of rat hepatocytes as we described previously) and to ELISA specific for HGF. The active fractions were pooled and concentrated to 1 mL by ultrafiltration (Amicon, Danvers, MA) using a YM-10 membrane filter. The purified HGF was used for further characterization such as treatment with *N*-glycanase (Genzyme Corp., Cambridge, MA) as recommended by the supplier.

Iodination of HGF. Baculovirus-expressed pro-HGF was purified from culture supernatants of infected insect cells grown in Ex-Cell 400 serum-free medium by FPLC–heparin–agarose chromatography as described above. Approximately 1 μg of pro-HGF was iodinated by the chloramine-T method (Higuchi & Nakamura, 1991). The iodinated HGF was then separated from free [^{125}I]Na by chromatography on a disposable Excellulose GF-5 column (Pierce, Rockford, IL). The specific activity of iodinated HGF was found to be 5×10^4 cpm/ng.

Preparation of Rat Liver and Spleen Extracts. Rats were anesthetized by injection of Nembutal sodium solution (Abbott Laboratories, North Chicago, IL), and their livers and spleens were removed and washed in cold PBS. Tissues were minced to small pieces and rinsed several times with large volumes of PBS. A 1-g sample of minced tissue was placed in 5 mL of PBS and homogenized with a polytron homogenizer set at high speed for 1 min. Tissue debris was removed by centrifugation at 1000*g* for 10 min. The supernatant from each sample was then centrifuged at 12500*g* for 20 min. The resulting supernatant (soluble extract) and pellet (particulate fraction) were used at 50 μg of total protein for incubation with baculovirus-expressed [^{125}I]HGF in 10-μL reactions. In some reactions PMSF was used at a final concentration of 5 mM. After incubation at 37 °C for 16 h, samples were subjected to SDS–PAGE followed by autoradiography.

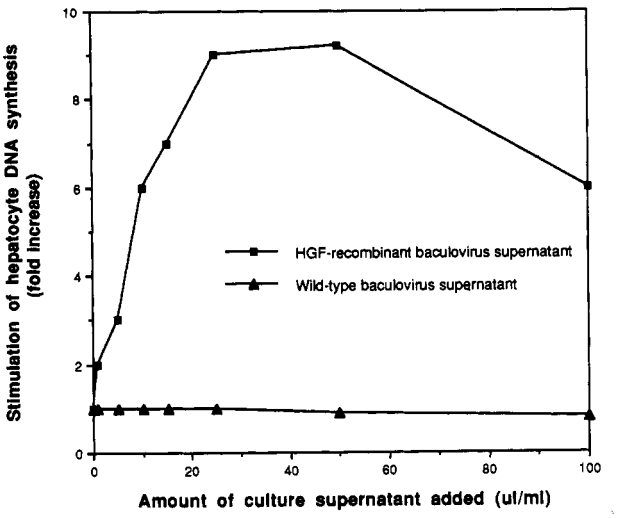

FIGURE 2: Secretion of bioactive HGF in the supernatant of insect cells infected with the recombinant baculovirus. Stimulation of DNA synthesis in primary cultures of rat hepatocytes by culture supernatants from Sf9 cells infected with either HGF-recombinant baculovirus (squares) or wild-type baculovirus (triangles; similar results were obtained with supernatants from uninfected cells and from culture medium alone). The data are presented as *x*-fold increases in DNA synthesis ([^{3}H]thymidine incorporation in triplicate cultures) over untreated cultures.

Assay for Stimulation of DNA Synthesis and Motility in Hepatocytes by Baculovirus-Expressed HGF. Rat, human, or mouse hepatocytes were isolated by collagenase perfusion and subjected to mitogenic assay ([^{3}H]thymidine incorporation) as we described previously (Zarnegar & Michalopoulos, 1989). A motility assay (migration from microcarrier beads) was performed essentially as described by Rosen et al. (Rosen et al., 1990) with the exception that hepatocytes were incubated with collagen-coated microcarrier beads for about 6 h, washed, and incubated in collagen-coated six-well plates in the presence or absence of HGF (50 ng/mL) or EGF (20 ng/mL) for 24–48 h. At the end of the incubation period, plates were washed three times with PBS and once with ice-cold 5% TCA solution. The remaining attached cells were enumerated using an inverted phase-contrast microscope, and the cell numbers per field (a total of 100 fields were counted) in each group were determined.

RESULTS AND DISCUSSION

Construction of Recombinant Baculovirus. A major aim of this study was to establish a system that would produce large quantities of bioactive human recombinant HGF. The insect cell-based recombinant baculovirus expression system has been reported to express large amounts of various cloned foreign gene products including human plasminogen cDNA (Davidson et al., 1990), which shares sequence and structural homology with hepatocyte growth factor (HGF) (Nakamura et al., 1989). To achieve this goal, we first cloned a full-length human HGF cDNA containing the signal peptide sequence, 26 nucleotides of the 5′ untranslated region, the entire coding region, and 84 nucleotides of the 3′ untranslated portion of the HGF sequence into the baculovirus transfer vector pBlueBac as described in Materials and Methods. A map of pBlueBac containing the HGF cDNA (pBlueBac–

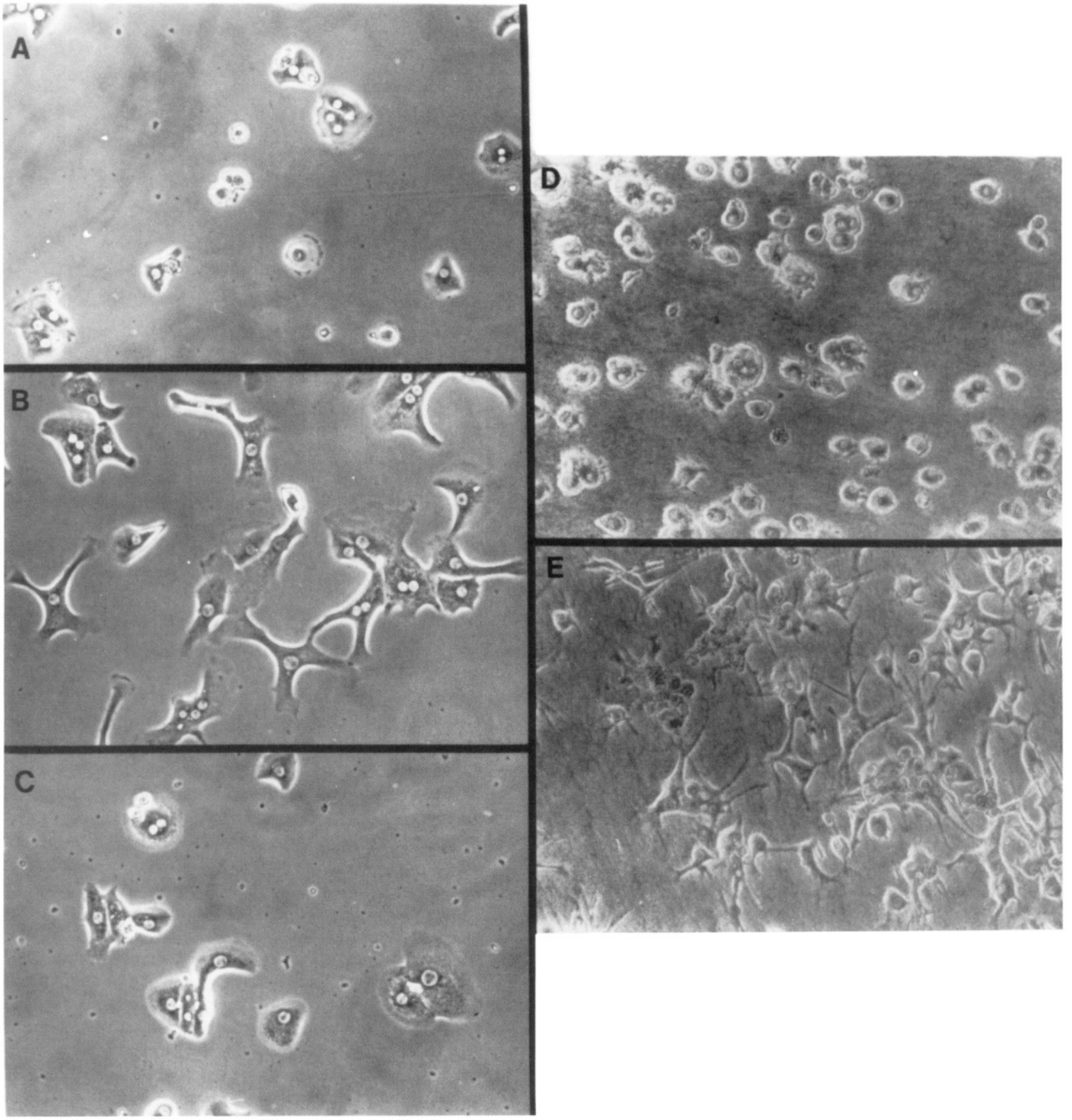

FIGURE 3: Induction of morphological change (scattering effect) in primary cultures of rat and human hepatocytes. Hepatocytes were isolated by collagenase perfusion and cultured in collagen-coated plates (frames A–C, rat hepatocytes) or in collagen gels (frames D and E, human hepatocytes) in the presence (frames B, C, and E) or absence (frames A and D) of baculovirus-expressed HGF. In frame C, baculovirus-expressed HGF was added in the presence of anti-HGF serum (1:500 final dilution). Photomicrographs were taken 24 h posttreatment.

HGF) is shown in Figure 1. Six days after cotransfection of Sf9 cells with the pBlueBac–HGF construct and wild-type baculovirus (AcNPV) DNA, the medium was harvested, and recombinant baculovirus containing the pBlueBac–HGF construct was isolated by plaque assay. The presence of the HGF cDNA insert in the genome of recombinant baculovirus and the expression of its mRNA was ascertained by PCR and RT–PCR, respectively, utilizing HGF-specific primers that produce an expected amplified product of approximately 700 bp (data not shown).

Production of Biologically Active Human HGF by Sf9 Cells Infected with HGF-Recombinant Baculovirus. Insect cells (Sf9 cells or High 5 cells purchased from Invitrogen) were infected with the HGF-recombinant baculovirus at an MOI of 2:1 and incubated for up to 7 days in Grace's insect medium containing 10% FBS. The culture supernatant was removed at 3, 5, and 7 days postinfection and subjected to bioassay for HGF (stimulation of DNA synthesis in primary cultures of rat hepatocytes). As depicted in Figure 2, HGF activity was detected in a dose-dependent fashion only in the culture supernatant of insect cells infected with the HGF-recombinant baculovirus. Significant stimulation of DNA synthesis was induced by as low as 1 μL of culture supernatant (1:1000 final dilution), reaching a maximum of 10-fold over the control at 5–30 μL/mL depending on the preparation. Supernatant from control cultures of insect cells infected with

wild-type AcNPV virus, no virus, or culture medium by itself lacked such activity (Figure 2).

When culture medium from recombinant baculovirus infected cells was subjected to bioassay as a function of time of infection, HGF activity was detected at day 3 postinfection and continued to increase as a function of time of culture. This indicates that recombinant HGF is synthesized, secreted, and accumulated in the culture medium. In addition, daily pulse-labeling of infected insect cells with L-[^{35}S]methionine from day 2 to day 7 postinfection and analysis of cell extract from these cells by SDS–PAGE and autoradiography indicate that HGF synthesis peaks at day 4 (see below). For large-scale production of HGF, cells are routinely infected for 6 days, resulting in a high yield of HGF of up to 5 mg/L.

We also examined the High 5 insect cell line which is known to be more efficient in the production of recombinant gene products than the Sf9 cells. Four-fold higher levels of bioactive human recombinant HGF were produced by these cells than Sf9 cells when infected with HGF-recombinant baculovirus (per cell basis, data not shown). The amount of recombinant HGF present in the culture supernatant of infected insect cells was determined by a specific ELISA utilizing chicken anti human HGF serum. The concentrations of immunoreactive HGF in the culture media of cells infected with HGF-recombinant baculovirus ranged from 3 to 8 mg/L depending on the MOI and insect cells (Sf9 cells versus High 5 cells). The potency of the purified recombinant HGF in stimulating DNA synthesis in a primary culture of rat hepatocytes is comparable to that of native HGF purified from human placenta or rabbit serum. Similar results were also obtained when primary cultures of human or mouse hepatocytes were used as target cells, indicating that the baculovirus-expressed human recombinant HGF, like the native HGF, is species nonspecific (data not shown).

As indicated earlier, another well-known effect of HGF on a variety of epithelial cells is its ability to dissociate and scatter monolayers of cells in culture and to stimulate their motility and migration (Gherardi & Stoker, 1990; Naldini et al., 1991; Weidner et al., 1991). We noted that native HGF induces a drastic morphological change in primary cultures of rat hepatocytes detectable at 16–24 h after plating (R. Zarnegar and G. Michalopoulos, personal communication, 1987). As shown in Figure 3, the baculovirus-expressed recombinant HGF also induces a pronounced change in the morphology of normal rat, mouse, and human hepatocytes (Figure 3B,E, rat and human hepatocytes, respectively), and this effect was totally abrogated by antiserum raised against HGF (Figure 3C). The morphological changes notable at 16–24 h after plating hepatocytes in the presence of HGF included formation of long processes, spreading, and scattering. It should be noted that EGF (epidermal growth factor, a potent mitogen for hepatocytes) induced similar morphological changes in these cells. Addition of baculovirus-expressed HGF to rat hepatocytes also drastically stimulates the migration of these cells from microcarrier beads onto collagen-coated plates (30-fold over untreated control cultures for HGF and 4-fold with EGF). These results indicate that HGF is a potent motogen for hepatocytes.

To prove that the growth-promoting activities that are present in the culture supernatant of insect cells infected with HGF-recombinant baculovirus are due to HGF, immunoneutralization studies were carried out utilizing HGF-specific polyclonal antiserums raised against human or rabbit HGF. As shown in Figure 4, the addition of either of these antiserums at a 1:500 dilution neutralizes the stimulatory activity

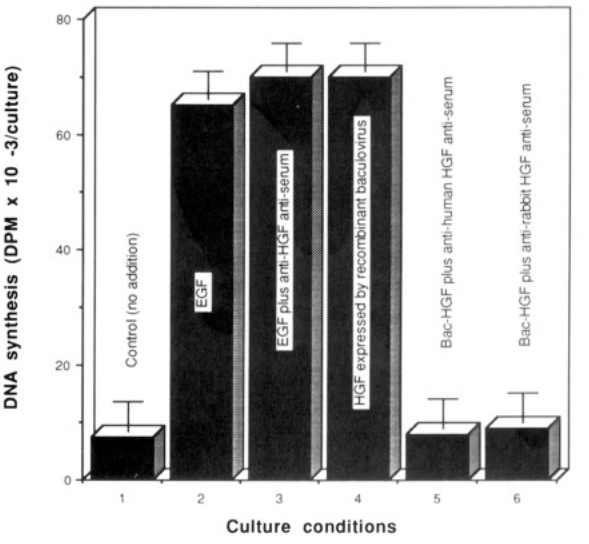

FIGURE 4: Immunoneutralization of HGF bioactivity secreted by baculovirus infected insect cells. Primary cultures of rat hepatocytes were incubated in the presence of 20 ng/mL EGF, or 10 μL of culture supernatant from Sf9 insect cells infected with HGF-recombinant baculovirus with or without chicken anti-HGF serums (1:500 final dilution) as indicated in the figure. DNA synthesis in triplicate cultures ([^{3}H]thymidine incorporation) was determined as described under Materials and Methods.

(mitogenic and motogenic effects) present in the supernatant of insect cells infected with HGF-recombinant baculovirus. These results confirm that the growth-promoting activity present in the culture supernatant of baculovirus infected insect cells is the product of the HGF cDNA expressed in these cells.

Characterization of Recombinant HGF Biosynthesis. As mentioned above, HGF purified from serum or aggregated platelets consists of a heterodimeric glycoprotein comprised of an α chain (heavy chain) and a β chain (light chain) with apparent M_r's of 58 000–69 000 and 30 000–34 000, respectively (Nakamura et al., 1987; Gohda et al., 1988; Zarnegar & Michalopoulos, 1989; Zarnegar et al., 1989). In contrast, HGF purified from serum-free conditioned medium of fibroblast cell lines, such as MRC-5 or recombinant HGF purified from serum-free culture medium of mammalian cells transfected with HGF cDNA, is composed of a mixture of a proteolytically processed heterodimeric form with subunits having M_r's of 62 000 and 32 000 (α and β chains, respectively) as well as the unprocessed single-chain pro-HGF with an M_r of about 90 000 (Weidner et al., 1990; Rubin et al., 1991).

To determine whether HGF produced by the HGF-recombinant baculovirus infected insect cells is proteolytically converted to mature heterodimeric HGF intracellularly or secreted into the culture medium as single-chain pro-HGF, we metabolically pulse-labeled the proteins synthesized by these cells with L-[^{35}S]methionine. The infected cells were pulsed for 15 min on day 4 postinfection, which corresponds to the peak of HGF protein synthesis, and the cell extracts and the culture supernatants were analyzed at the indicated chase period. At the end of each pulsing period, the cell extract was subjected to SDS–PAGE and autoradiography or Western immunoblotting. Cell extracts and culture supernatants from metabolically labeled insect cells infected with wild-type baculovirus or no virus were also analyzed in parallel experiments.

As shown in Figure 5, only extract from cells infected with HGF-recombinant baculovirus contained a prominent radiolabeled (Figure 5A, lanes 2–6) and immunoreactive band with

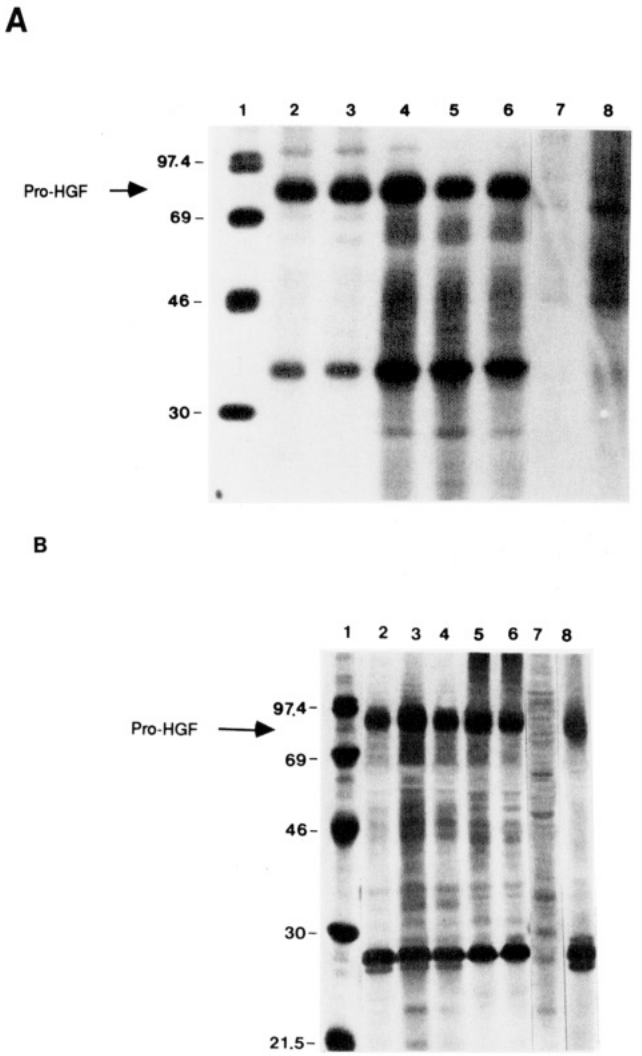

FIGURE 5: SDS–PAGE analysis of HGF expressed by recombinant baculovirus infected insect cells. (A) Sf9 insect cells were infected with HGF-recombinant baculovirus (lanes 2–6) or wild-type baculovirus (lane 8), or cultured in the absence of any virus (lane 7). On day 4 postinfection, cells were pulsed with L-[^{35}S]methionine for 15 min and chased with Grace's insect medium containing 90 μg/mL unlabeled methionine for different times (15 min, 2 h, 4 h, 8 h, and 24 h; lanes 2–6, respectively). Cell extracts were prepared for SDS–PAGE analysis and autoradiography as described under Materials and Methods. (B) Western immunoblot analysis of Sf9 infected insect cell extract on day 4 postinfection with HGF-recombinant baculovirus analyzed under reducing (lanes 2–6) conditions. Lane 8 shows the same sample as in lane 2 analyzed under nonreducing conditions. Cell extract from Sf9 cells infected with wild-type AcNPV is shown in lane 7. Samples in lanes 2–6 correspond to different time points after chase as indicated for (A). The migration position of ^{14}C-labeled molecular size standards (lane 1) are indicated in the figure. Rainbow protein molecular weight markers (Amersham) are myosin, MW 200 000; phosphorylase b, MW 97 400; bovine serum albumin, MW 69 000; ovalbumin, MW 46 000; carbonic anhydrase, MW 30 000; trypsin inhibitor, MW 21 500; and lysozyme, MW 14 300. The migration position of pro-HGF is indicated by an arrow.

an M_r of 90 000 under reducing conditions (Figure 5B, lanes 2–6). The migration position of this radiolabeled band did not change to a lower position for up to 24 h of chase with unlabeled methionine, indicating a lack of proteolytic conversion of pro-HGF to heterodimer. In addition to the M_r 90 000 band, a radiolabeled band with an M_r of 36 000 and a prominent immunoreactive band with an M_r of 28 000 were seen only in the recombinant baculovirus infected insect cell extract (Figure 5). These bands were present even if samples were run under nonreducing conditions and may represent fragments of HGF derived by proteolytic degradation.

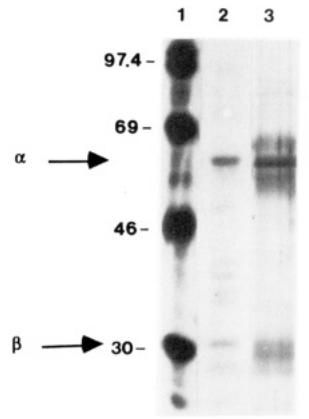

FIGURE 6: SDS–PAGE analysis of HGF secreted by HGF baculovirus infected insect cells grown in Grace's medium containing 10% FBS. Culture supernatant from Sf9 cells infected with HGF-recombinant baculovirus and pulsed with L-[^{35}S]methionine for 4 h, as described in the legend of Figure 5A, was analyzed by SDS–PAGE under reducing conditions and subjected to autoradiography (lane 2, 5-day exposure). Lane 3 shows the silver-stained pattern of bioactive baculovirus-expressed HGF purified from culture supernatant of HGF baculovirus infected insect cells (day 6 postinfection) by heparin-affinity chromatography. α and β refer to the heavy and light chains of HGF, respectively.

Analysis of the culture supernatants from metabolically radiolabeled infected insect cells grown in 10% FBS by SDS–PAGE and autoradiography, as well as fractionation of the culture supernatant by heparin–agarose-affinity chromatography and silver staining of the bioactive purified product, indicates that in the culture medium the secreted HGF is only present as the mature heterodimeric form with M_r's of 62 000 (α chain) and 30 000–32 000 (β chain doublet, Figure 6, lanes 2 and 3), and no pro-HGF is detectable. Under nonreducing conditions only a diffuse band with an M_r of 60 000 was noted, and no β chain was present, indicating that the β chain is covalently linked to the α chain (via disulfide bonds) as expected. The anomalous migration (shift to higher molecular mass after reduction) of HGF in SDS–PAGE under nonreducing conditions is due to the presence of several intrachain disulfide bonds (i.e., four kringle domains each having three disulfide bonds; Nakamura et al., 1989) and was previously reported by us (Zarnegar & Michalopoulos, 1989).

To investigate whether serum, which is added to the insect culture medium (Grace's insect medium), is a source of enzyme(s) for processing the secreted recombinant baculovirus-expressed pro-HGF, we grew Sf9 and High 5 insect cells in Ex-Cell 400 serum-free medium and then infected them with the HGF-recombinant baculovirus. Six days postinfection, large amounts of bioactive HGF were recovered from this serum-free system (data not shown). Analysis of the recombinant HGF secreted into the Ex-Cell 400 serum-free culture medium demonstrated that HGF is present in the medium mainly as single-chain pro-HGF with an M_r of approximately 90 000 (see Figure 7A, lane 1).

Incubation of radio-iodinated single-chain pro-HGF with freshly isolated rat serum or different amounts of FBS in a cell-free system resulted in a clear conversion of pro-HGF to α and β chains (Figure 7A, lanes 3–5, respectively). The conversion of pro-HGF to the mature heterodimeric form by serum was substantially reduced by PMSF, indicating the involvement of serine protease(s) in this process (Figure 7A, lane 2).

Recent *in vitro* mutagenesis studies (in which the Arg^{494}–Val^{495} cleavage site was mutated) have indicated that although single-chain pro-HGF binds to its receptor (c-MET) on target

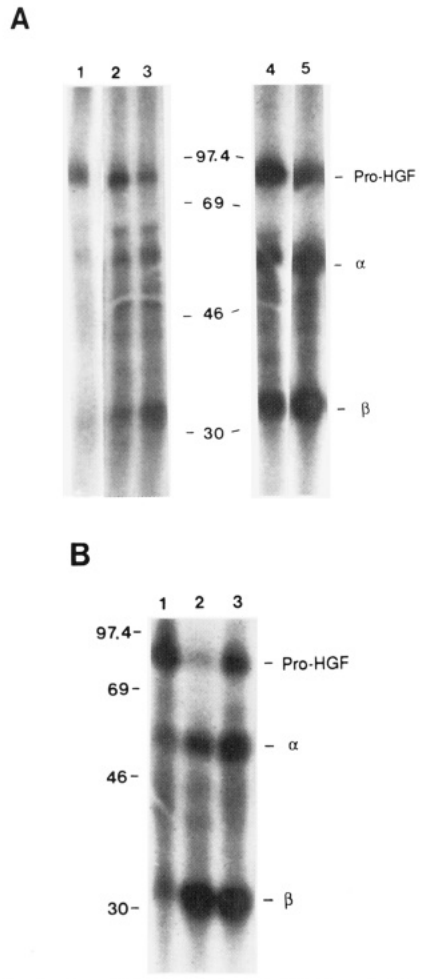

FIGURE 7: Activation of single-chain pro-HGF to the heterodimeric form by liver and spleen extracts. (A) Baculovirus-expressed single-chain pro-HGF (radio-iodinated, 0.5 ng in 5 μL) was incubated in PBS (lane 1) or with 1% rat serum (lanes 2 and 3) or with FBS at 5% and 10% (lanes 4 and 5, respectively) in a total volume of 10 μL. The sample in lane 2 was incubated in the presence of 5 mM PMSF. (B) [^{125}I]-pro-HGF was incubated in PBS alone or in PBS containing extracts from liver and spleen as described under Materials and Methods (lanes 2 and 3, respectively). Lane 1 represents radio-iodinated single-chain pro-HGF incubated with PBS. The reaction mixture contained 50 μg of total protein from each tissue extract plus 0.5 ng of radio-iodinated HGF in a total volume of 10 μL. The reactions were incubated at 37 °C for 16 h and then subjected to SDS–PAGE and autoradiography.

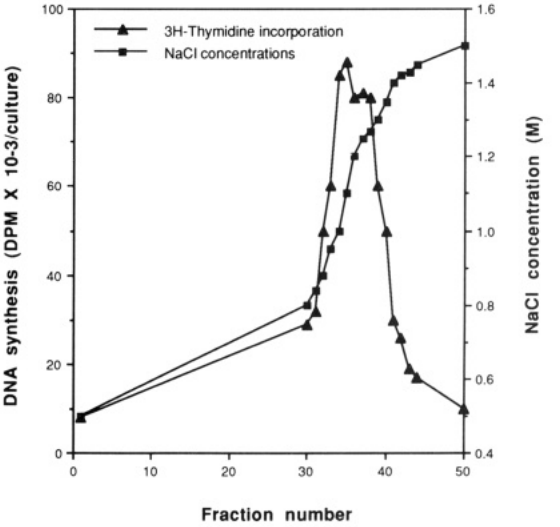

FIGURE 8: Purification of baculovirus-expressed recombinant HGF by heparin–agarose-affinity chromatography. Approximately 100 mL of supernatant from insect cells infected with HGF-recombinant baculovirus (grown in Ex-Cell 400 serum-free medium) was adjusted to 0.4 M NaCl and applied to a heparin–agarose column attached to an FPLC apparatus (Pharmacia). After washing the column with 700 mL of 0.5 M NaCl solution, the bound material was eluted with a linear gradient of 0.5–1.5 M NaCl at a flow rate of 1 mL/min. Fractions of 2 mL were collected, and 2 μL from each fraction was added to primary cultures of rat hepatocytes plated and cultured under serum-free conditions. DNA synthesis in triplicate cultures was determined as described under Materials and Methods.

cells, it does not stimulate the tyrosine kinase activity of c-MET (Matsumoto et al., 1991c; Gak et al., 1992; Hartmann et al., 1992; Lokker et al., 1992); hence, it is not biologically active. These studies have shown that specific proteolytic cleavage at the Arg^{494}–Val^{495} site is required for further transmission of the signals elicited by the binding of HGF to its receptor. We were interested in determining whether the baculovirus-expressed single-chain pro-HGF could induce DNA synthesis in primary cultures of rat hepatocytes. To eliminate serum as the possible source of pro-HGF activation, we plated rat hepatocytes in the absence of serum and incubated them with various amounts of pure single-chain pro-HGF as described under Materials and Methods. Addition of 20 ng/mL pure pro-HGF to these hepatocytes resulted in a 10-fold increase in DNA synthesis (data not shown), indicating that hepatocytes can activate single-chain pro-HGF to the active heterodimer. We incubated ^{125}I-labeled pro-HGF with extracts prepared from rat liver and spleen to determine whether these tissues could convert pro-HGF to the heterodimer. Figure 7B, lanes 2 and 3, shows that soluble extract from liver and spleen were able to convert the single-chain pro-HGF to the heterodimer. Further investigation is required to identify and characterize the enzyme(s) responsible for pro-HGF cleavage and to determine their exact cellular localization.

A recent study reported that a preparation of HGF consisting of a mixture of single-chain pro-HGF (the main component) and the mature heterodimeric HGF can stimulate DNA synthesis in primary cultures of rat hepatocytes to the same extent as that of pure mature heterodimeric HGF (Naka et al., 1992). These studies also demonstrated that the addition of serine protease inhibitors diminished the mitogenic effects observed in the cultures treated with pro-HGF. In addition, these researchers showed that the incubation of [^{125}I]HGF with primary cultures of rat hepatocytes resulted in the conversion of pro-HGF to the heterodimer. On the basis of their observations, it was suggested that cultured hepatocytes are able to proteolytically convert pro-HGF to the bioactive mature heterodimeric form (Naka et al., 1992). The interpretation of these results was complicated by the fact that the preparation of pro-HGF used in their studies contained substantial amounts of the heterodimeric form and also by the fact that rat hepatocytes used as the target cells were initially plated in serum-containing medium (4-h plating period), which could potentially be a source for pro-HGF activation. The results of our findings, however, support the notion that cultured hepatocytes themselves can activate the pro-HGF to the bioactive form in the absence of serum and that liver contains the necessary enzyme(s) to cleave pro-HGF to the heterodimer.

Other studies have shown that the mitogenic effects of single-chain pro-HGF on B5/589 (human mammary epithelial cell line) were inhibited by the protease inhibitor aprotinin. Yet when single-chain pro-HGF was treated with plasmin prior to addition to these cells (in the presence of protease inhibitor), it was fully active, supporting the idea that proteolytic cleavage had occurred during the bioassay and is required for biological activity of pro-HGF (Gak et al., 1992). No structural analysis

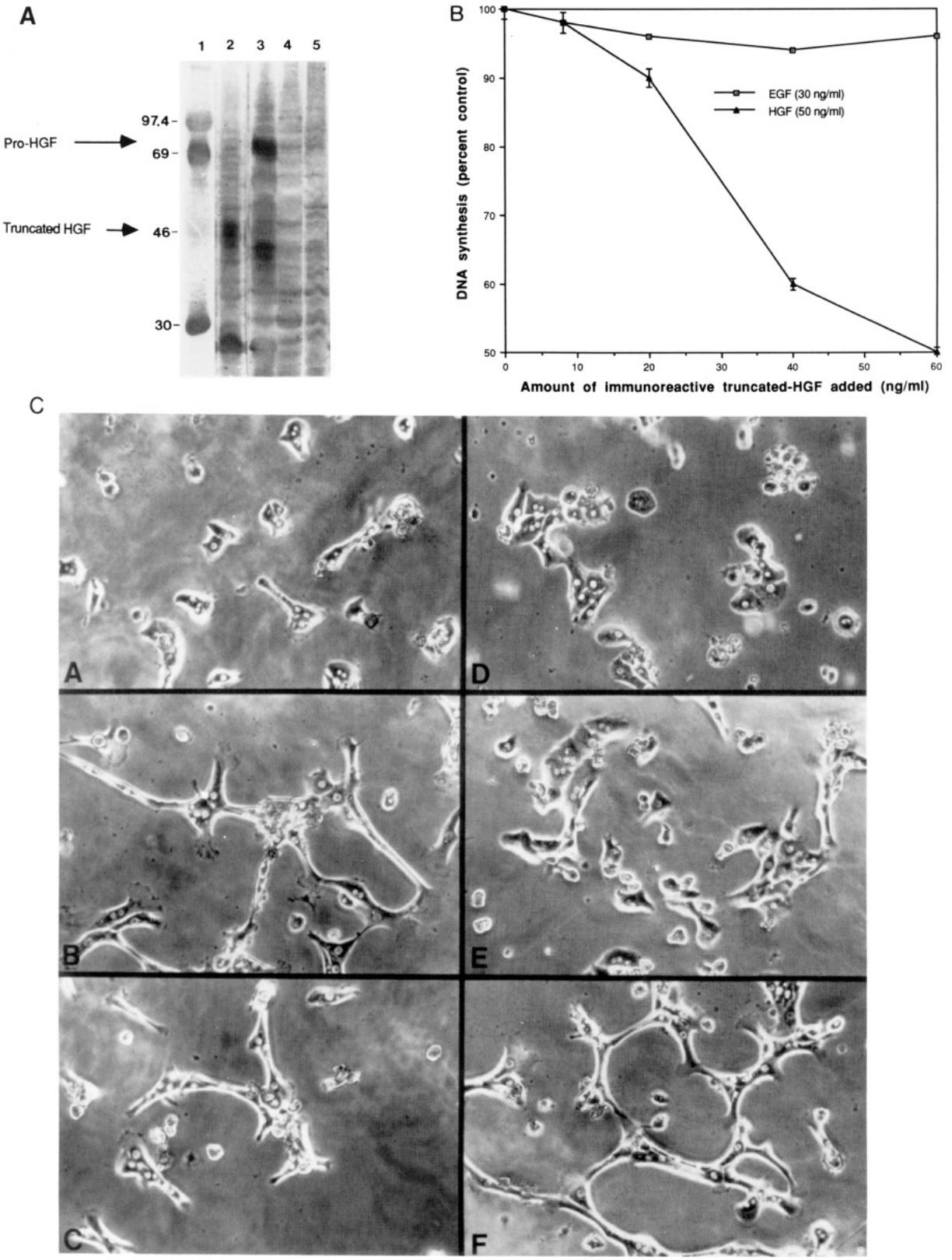

FIGURE 9: Antagonistic effect of baculovirus-expressed truncated HGF on DNA synthesis induced in hepatocytes by full-length recombinant HGF. A mutant form of HGF containing the N-terminal portion of HGF (kringles 1–3) was expressed in the baculovirus expression system as described under Materials and Methods. (A) Western immunoblot analysis of cell extract from Sf9 cells infected with the recombinant baculovirus containing the truncated HGF cDNA (lane 2), full-length HGF cDNA (lane 3), wild-type baculovirus (lane 4), or Sf9 cell extract grown in the absence of any virus (lane 5). Lane 1 shows the migration position of the rainbow molecular size markers. (B) Increasing amounts of the truncated HGF expressed by recombinant baculovirus (supernatant from 6 days postinfection) were added to primary cultures of rat hepatocytes in the presence of 30 ng/mL EGF (squares) or 50 ng/mL full-length baculovirus-expressed HGF (triangles). EGF or HGF at these doses induced DNA synthesis to about 10-fold over that of untreated control cultures. The truncated HGF alone (in the absence of HGF or EGF) did not stimulate any significant increase in DNA synthesis above untreated control cultures. The concentration of the truncated HGF was approximately 5 μg/mL as determined by ELISA using a polyclonal antiserum against full-length human HGF. (C) Inhibition of HGF induced morphological changes and scattering of hepatocytes by the truncated HGF. Frame A represents untreated control rat hepatocytes 24 h in culture. Frames B and D are cultures treated with 50 ng/mL full-length or 100 ng/mL truncated HGF, respectively. Frame C shows cells treated with 30 ng/mL EGF only. Frame E represents cells exposed to 100 ng/mL truncated HGF plus 50 ng/mL full-length HGF, and frame F shows cultures treated with 100 ng/mL truncated HGF plus 30 ng/mL EGF.

(SDS–PAGE) of the plasmin-treated pro-HGF was presented by these researchers, thus making it difficult to assume that cleavage had resulted in the formation of α and β chains. These researchers also claim that plasminogen activators (u-PA) failed to cleave pro-HGF to the heterodimeric form (Gak et al., 1992). On the other hand, Mars et al. (1993) reported that incubation of [^{125}I]HGF with u-PA causes the conversion of single-chain pro-HGF to the heterodimer. As can be concluded from the results published in the literature at the present time, the physiological enzyme(s) that activates pro-HGF remains to be identified. The baculovirus-expressed single-chain pro-HGF we have prepared and used in our current studies has the advantage of existing only in the single-chain pro-HGF form and will be useful for further characterization of the proteolytic enzyme(s) involved in HGF activation.

Further Characterization of Recombinant HGF. The baculovirus-expressed recombinant HGF retained most of the biochemical and structural characteristics of native HGF purified from different sources such as serum and plasma (Nakamura et al., 1987; Gohda et al., 1988; Zarnegar & Michalopoulos, 1989; Zarnegar et al., 1989), human placenta (Hernandez et al., 1992), conditioned medium of the human embryonic lung fibroblast cell line MRC-5 (Weidner et al., 1990; Rubin et al., 1991), or recombinant HGF purified from the culture supernatant of mammalian cells stably transfected with a human HGF cDNA. Among these properties are N-glycosylation and the ability to bind to heparin. As shown in Figure 8, the baculovirus-expressed recombinant HGF bound to immobilized heparin and eluted from this matrix with the same NaCl concentration as reported for native HGF (Nakamura et al., 1987; Gohda et al., 1988; Zarnegar & Michalopoulos, 1989; Zarnegar et al., 1989). Treatment of the purified baculovirus-expressed recombinant HGF with *N*-glycanase reduced the M_r of the α chain (with an M_r of about 60 000) to a band with a molecular mass of 50 000, and the M_r of the β chain doublet to the M_r of 29 000. Treatment of native HGF, purified from human placenta, with *N*-glycanase resulted in a similar pattern (data not shown).

Expression and Characterization of the Truncated Form of HGF. As described under Materials and Methods, we generated a deleted form of the HGF cDNA, encoding only the amino terminal portion of HGF (kringles 1–3 and part of kringle 4). A truncated, naturally occurring variant form of HGF containing kringles 1 and 2 with an M_r of 28 000 (p28) was originally copurified with full-length HGF from culture supernatants of MRC-5 fibroblast cells (Chan et al., 1991). Its cDNA was cloned and sequenced from cDNA libraries prepared from fibroblast cells (Rubin et al., 1991) and human placenta (Miyazawa et al., 1991), and the truncated form of HGF was determined to represent a splice variant of HGF. Although the truncated protein binds to the HGF receptor, c-MET (Bottaro et al., 1991; Chan et al., 1991), it does not stimulate tyrosine kinase activity of the receptor and lacks any detectable biological activities (mitogenic or motogenic) (Chan et al., 1991; Matsumoto et al., 1991; Okigaki et al., 1992). It was suggested that p28 acts as a natural antagonist for HGF and modulates HGF activity (Chan et al., 1991).

We produced a truncated form of HGF cDNA and expressed it in the baculovirus expression system as described above for the full-length form of HGF. This mutant HGF is recognized by polyclonal antiserum raised against human HGF as determined by Western immunoblot and ELISA (the concentration of truncated HGF ranges from 3 to 5 mg/L as determined by ELISA). Truncated HGF, which lacks the β chain, migrates with an apparent M_r of 47 000 (Figure 9A) as predicted from its cDNA sequence (see Materials and Methods) and appears to have no biological activity (stimulation of DNA synthesis or induction of scattering and morphological changes) on rat hepatocytes by itself (Figure 9B,C, respectively). It competes, however, with full-length HGF and abrogates the mitogenic (Figure 9B) and morphological changes (Figure 9C) induced by full-length HGF.

Several independent studies using *in vitro* mutagenesis of HGF cDNA and transient expression systems to produce the mutated HGF have shown that the N-terminal hairpin loop or kringle 1 or 2 of HGF is necessary but not sufficient for HGF's biological activity (Matsumoto et al., 1991c; Lokker et al., 1992; Okigaki et al., 1992). Specifically, it appears that these domains are required for binding of HGF to its receptor. Deletion of kringle 3 or 4 did not have significant effects on the binding and mitogenic properties of HGF (Matsumoto et al., 1991c; Lokker et al., 1992; Okigaki et al., 1992). These studies also showed that the β chain of HGF, though not required for HGF binding to its receptor, is necessary for receptor activation and the mitogenic and motogenic effects of HGF. However, a recent paper appears to contradict some of the findings mentioned above. It was demonstrated in this report that the naturally occurring truncated variant of HGF, p28 (encoding the first and the second kringles), or an *in vitro* mutagenized form of HGF encoding only the α chain (kringles 1–4) binds to the HGF receptor on MDCK cells, induces its tyrosine kinase activity, and causes scattering of these cells. On the other hand, these forms of HGF were unable to stimulate mitogenesis in primary cultures of rat hepatocytes. The researchers concluded that the α chain of HGF by itself is sufficient for the motogenic but not the mitogenic effects of HGF (Hartmann et al., 1992). Further investigations are in progress in our laboratory to discern the effects of the baculovirus-expressed truncated HGF on the scattering of MDCK cells, its binding characteristics to the HGF receptor, and the activation of c-MET tyrosine kinase activity on these cells as well as on hepatocytes. The availability of baculovirus-expressed wild-type and mutant forms of HGF in milligram quantities will facilitate future investigations on this interesting growth factor, including its potential therapeutic efficacy and value.

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