

Heparin Induces Dimerization and Confers Proliferative Activity onto the Hepatocyte Growth Factor Antagonists NK1 and NK2

Ralph H. Schwall, Ling Y. Chang, Paul J. Godowski, David W. Kahn, Kenneth J. Hillan, Kenneth D. Bauer, and Thomas F. Zioncheck

Genentech, Inc., South San Francisco, California 94080-4990

Abstract. Hepatocyte growth factor (HGF) is a potent epithelial mitogen whose actions are mediated through its receptor, the proto-oncogene c-Met. Two truncated variants of HGF known as NK1 and NK2 have been reported to be competitive inhibitors of HGF binding to c-Met, and to function as HGF antagonists (Lokker, N.A., and P.J. Godowski. 1993. *J. Biol. Chem.* 268: 17145–17150; Chan, A.M., J.S. Rubin, D.P. Bottaro, D.W. Hirschfield, M. Chedid, and S.A. Aaronson. 1991. *Science (Wash. DC)*. 254:1382–1387). We show here, however, that NK1 acts as a partial agonist in mink lung cells. Interestingly, NK1, which is an HGF antagonist in hepatocytes in normal conditions, was converted to a partial agonist by adding heparin to the culture medium. The interaction of NK1 and heparin was further studied in BaF3 cells, which express little or no cell surface heparan sulfate proteoglycans. In BaF3 cells transfected with a plasmid encoding human c-Met, heparin and NK1 synergized to stimulate DNA synthesis and cell proliferation. There was no effect of heparin on the IL-3 sensitivity of BaF3-hMet cells, and no effect of

NK1 plus heparin in control BaF3 cells, indicating that the response was specific and mediated through c-Met. The naturally occurring HGF splice variant NK2 also stimulated DNA synthesis in mink lung cells and exerted a heparin-dependent effect on BaF3-hMet cells, but not on BaF3-neo cells. The activating effect of heparin was mimicked by a variety of sulfated glycosaminoglycans. Mechanistic studies revealed that heparin increased the binding of NK1 to BaF3-hMet cells, stabilized NK1, and induced dimerization of NK1. Based on these studies, we propose that the normal agonist activity of NK1 and NK2 in mink lung cells is due to an activating interaction with an endogenous glycosaminoglycan. Consistent with that model, a large portion of the NK1 binding to mink lung cells could be blocked by heparin. Moreover, a preparation of glycosaminoglycans from the surface of mink lung cells induced dimerization of NK1. These data show that the activity of NK1 and NK2 can be modulated by heparin and other related glycosaminoglycans to induce proliferation in cells expressing c-Met.

HEPATOCYTE growth factor (HGF)¹ was isolated as a hepatocyte-specific mitogen but has subsequently been found to stimulate proliferation of many types of epithelial cells, as well as vascular endothelial cells (17, 21). Although it acts on a broad range of epithelial cells, HGF has no activity on stromal cells. In fact, stromal cells produce HGF in many tissues containing HGF-sensitive epithelial cells, raising the possibility that HGF may be important in local stromal-epithelial communication (31, 34). In addition to its mitogenic activity, HGF stimulates cellular motility, which can be manifested as the “scattering” of cells that normally grow in tight clusters or

as enhanced movement across membranes in Boyden chamber assays (8, 35, 36). HGF is also cytotoxic for some sarcoma cells (11) and induces branching morphogenesis in certain cell types when these cells are cultured within collagen gels (20).

Both the mitogenic and motogenic responses to HGF are mediated through its receptor c-Met, a transmembrane tyrosine kinase (2, 22, 23, 37). C-Met is synthesized as a 170-kD precursor that is glycosylated to form a 190-kD species, and then proteolytically cleaved into two subunits (9). The larger β subunit contains the intracellular domain, with the tyrosine kinase and numerous docking sites for intracellular mediators, a single membrane-spanning domain, and part of the extracellular domain. The α subunit is exclusively extracellular and is coupled to the β subunit by disulfide bond.

Because c-Met is a proto-oncogene, there has been interest in the role of HGF and c-Met in cancer. Several different epithelially derived tumors (4–6, 15, 24, 28, 40) and

Address all correspondence to Ralph H. Schwall, Molecular Oncology, Genentech, Inc., 460 Point San Bruno Blvd., Mail Stop 40, South San Francisco, CA 94044. Tel.: (415) 225-1149. Fax: (415) 225-6497. e-mail: rhs@gene.com.

1. *Abbreviations used in this paper:* GAG, glycosaminoglycan; HGF, hepatocyte growth factor.

sarcomas (29, 30) have been found to overexpress c-Met protein. In addition, HGF stimulates proliferation of mammary epithelial cells (33), and high HGF levels in primary breast cancers have been associated with faster relapse and shortened survival time (38).

Full-length HGF is a 90-kD protein composed of an NH₂-terminal domain, four consecutive kringle domains, and a carboxy-terminal protease-like domain, and two truncated variants of HGF have been described as competitive HGF antagonists. The first variant, designated NK2 because it consists of the HGF NH₂-terminus through kringle 2, is the naturally occurring product of an alternatively spliced HGF mRNA (3). NK2 failed to stimulate proliferation in B5/589 mammary epithelial cells, but it bound to c-Met and blocked the mitogenic response to full-length HGF (3). Hartmann et al. confirmed that NK2 lacks mitogenic activity but found that it retained the ability to induce scattering in MDCK cells (10). These investigators suggested that the interaction of NK2 with c-Met is sufficient to activate the intracellular signaling required for motility but that full-length HGF is required to activate those signals involved in mitogenesis. NK2 was discovered in medium conditioned by SK-LMS-1 cells, but the same alternatively spliced transcript has been identified in normal human placenta, suggesting that NK2 has a physiological function (18).

More recently, Lokker and Godowski showed that a shorter HGF variant, truncated after the first kringle domain (amino acids 32–210) and designated NK1, also blocks binding of HGF to c-Met and is a potent inhibitor of HGF-induced DNA synthesis in rat hepatocytes (16). However, they also found that high concentrations of NK1 induced a small but detectable increase in tyrosine phosphorylation of c-Met in A549 cells, consistent with a weak agonist activity.

We have studied NK1 further to assess its potential as an anticancer agent, and we report here that the nature of the response to NK1 varies in different cell types. We show that NK1 is an HGF antagonist in hepatocytes, as described previously (16), but that in mink lung cells, NK1 has a direct stimulatory effect on DNA synthesis. To understand the discrepancy between hepatocytes and mink lung cells, we examined the effects of heparin on NK1 activity because NK1, like full-length HGF, binds heparin (16), and heparin promotes dimerization of HGF and augments the response to low doses of HGF in hepatocytes (41). In addition, heparan sulfate proteoglycans are important for the activity of FGF and other heparin binding growth factors (13, 32). We show here that there is a synergistic interaction of heparin with NK1 to stimulate DNA synthesis in cells expressing c-Met. Similar results were obtained with the naturally occurring variant NK2. Data such as these lead us to propose that glycosaminoglycans on the cell surface can interact with NK1 and NK2 to promote proliferative activity through c-Met, turning these variants from antagonists to agonists.

Materials and Methods

Materials

Recombinant human HGF was produced from medium conditioned by a

mammalian cell line derived by stable transfection with a full-length HGF cDNA, as previously described (41). NK1 was produced in *Escherichia coli* and purified as described by Lokker et al. (16). NK2 was produced by transient transfection in 293 cells with a cDNA corresponding to the sequence of human NK2 described by Chan et al. (3). Conditioned medium was passed over S-Sepharose and washed with 0.25 M NaCl, 20 mM Tris, pH 7.5, and NK2 was eluted by a NaCl gradient from 0.25 to 1.5 M into tubes containing 1 mg/ml BSA as carrier. Fractions that contained NK2 were identified by ELISA (see below) and pooled.

IL-3 was obtained from Genzyme Corp. (Boston, MA). Heparin, de-N-sulfated heparin, dextran sulfate, pentosan polysulfate, heparan sulfate, chondroitin sulfate, and sodium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Phycoerythrin-streptavidin was purchased from Jackson ImmunoResearch Laboratories (Avondale, PA). Bis(sulfosuccinimidyl) suberate (BS³) was purchased from Pierce Chemical Co. (Rockford, IL).

NK1/NK2 ELISA

A sandwich ELISA for NK1 was developed using two Abs against NK1 (kindly provided by Dr. Jin Kim and Kelly Tabor, Genentech Inc., South San Francisco, CA). Microtiter plates were coated overnight at 4°C with 2 µg/ml antibody 8A8 in sodium carbonate buffer, pH 9.6. The plate was then blocked with PBS, 0.5% BSA, 0.01% thimerosal (150 µl) for 2 h. This and all subsequent steps were performed at room temperature on an orbital shaker. The blocking buffer was discarded, the plate was washed three times with PBS, 0.05% Tween-20, 0.01% thimerosal, and the sample was incubated for 2 h. The plate was again washed three times and incubated for 2 h with biotinylated anti-NK1 antibody 6D5. The plate was then washed three times, incubated with HRP-streptavidin (Zymed Laboratories, Inc., South San Francisco, CA) for 30 min, and washed three more times. *O*-phenylene diamine and H₂O₂ were added, and color was allowed to develop for 10–20 min. The reaction was stopped with sulfuric acid and absorbance at 492 nm, less the absorbance at the reference wavelength of 405 nm, using a microtiter plate reader. This assay recognizes NK1 and NK2.

Hepatocytes and Mink Lung Cells

Hepatocytes were isolated from adult female Sprague-Dawley rats by collagenase perfusion, according to the methods of Garrison and Haynes (7). The cells were washed three times with William's E medium supplemented with 10 µg/ml transferrin, 1 µg/ml insulin, 1 mg/ml BSA, 50 µg/ml Gentamycin, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin, resuspended in the same medium, and then seeded into a 96-well plate (5,000 cell per well) with the factors to be tested. 16 h later, 1 µCi [³H]thymidine was added, and after an additional 24 h, the cells were transferred onto plates with a cell harvester (Unifilter GF/B, Filtermate 196; Packard Instrument Co., Inc., Downers Grove, IL). The amount of radioactivity incorporated into DNA was measured in a microplate scintillation counter (Top Count; Packard Instrument Co., Inc.).

Mink lung cells were passaged in MEM, 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. To measure DNA synthesis, the cells were released from the flask with trypsin-EDTA, washed twice with serum-free assay medium (DME/F12 (50:50)), 2 mM glutamine, and 1 mg/ml BSA, and seeded into 96-well plates (10,000 cells per well) containing the factors to be tested. DNA synthesis was then measured as described above for hepatocytes.

BaF3-hMet Cells

A full-length cDNA for human c-Met was inserted into the pRK5-tk.neo vector. The resulting plasmid was linearized and introduced into BaF3 cells by electroporation at 250 V, 800 µF. Controls were transfected with the empty vector. Transformants were selected in 2 mg/ml G418, cloned by limiting dilution, and the resulting cell lines were tested for their ability to respond to HGF, as described below. Those cell lines that responded to HGF were also found to express c-Met by Western blotting (data not shown).

Parental BaF3 and the transfected sublines were passaged in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, β-mercaptoethanol (4 µl/L), and 5% WEHI-conditioned medium as a source of IL-3. Transfected sublines were maintained in 1 mg/ml G418. To measure DNA synthesis, the cells were washed twice with RPMI 1640 containing 10% FBS and glutamine, but no IL-3, and resuspended in the same medium; then

5,000 cells per well were added to a 96-well plate containing the factors to be tested. The following morning, 1 μCi [^3H]thymidine was added, and the amount incorporated into DNA was measured 7 h later. To measure cell growth, cells were seeded in a 24-well plate in 1 ml of the above medium containing the factors to be tested, but no IL-3, and 7 d later, cells were counted using a particle counter (ZM; Coulter Corp., Hialeah, FL).

Binding Analysis

BaF3-hMet cells were washed, resuspended in cold Dulbecco's PBS, 0.5% BSA, and then incubated with increasing concentrations of NK1 (0.1–20 nM) in the absence and presence of 1 $\mu\text{g}/\text{ml}$ heparin for 1 h at 4°C. The cells were then washed twice and resuspended in buffer containing 2 $\mu\text{g}/\text{ml}$ biotinylated mAb 6D5. After a 30-min incubation, the cells were washed and resuspended in 50 μl phycoerythrin-streptavidin (final dilution 1:50). After an additional 30 min on ice, the cells were washed and resuspended in buffer. Fluorescence was quantitated by flow cytometry, using an EPICS Elite instrument (Coulter Corp.) equipped with an argon ion laser. Orange fluorescence at 575 ± 15 nm was collected after 488-nm laser (15 mW) excitation. 10,000 cells were analyzed, and the mean fluorescent intensity was calculated. Background fluorescence in the absence of NK1 was subtracted.

Dimerization

The cross-linking reagent bis(sulfosuccinimidyl) suberate was added (1 mM final concentration) to a tube containing 1 μg NK1 with or without 1 μg heparin in a total volume of 20 μl of 0.15 M NaCl. After 20 min, the reaction was quenched by addition of 5 μl of 1 M Tris-HCl, pH 6.8. SDS-PAGE sample buffer with 10 mM β -mercaptoethanol was added, and then the samples were heated at 100°C for 7 min and loaded onto a 4–20% SDS gel. After electrophoresis, bands containing NK1 were identified by Western blotting with a cocktail of anti-NK1 mAbs, followed by HRP-sheep anti-mouse IgG (Amersham Corp., Arlington Heights, IL) and detection with the SuperSignal CL kit (Pierce Chemical Co.).

Dimerization was confirmed by testing the cross-linked products in a dimer-specific NK1 ELISA. The assay was performed as described above, except that the plate was coated with antibody 6D5 rather than 8A8. In this format, the same antibody is used both for capture and detection. Monomeric NK1 is not detected because binding to the capture antibody blocks the epitope from the detection antibody, but dimeric NK1 presents two epitopes, allowing a signal to be generated. Samples to be tested were cross-linked as described above, and then diluted to 1 ml with 0.5 M NaCl to minimize nonspecific aggregation.

Preparation of Mink Lung Cell Surface Glycosaminoglycans

To release cell surface glycosaminoglycans (GAGs), six confluent 150-mm plates of mink lung cells were rinsed with PBS and incubated with 2 ml trypsin-EDTA at 37°C until the cells detached. The trypsin solution was then collected, pooled, and after removing the cells by centrifugation, heated in a boiling water bath for 30 min to denature the trypsin. The GAGs were then partially purified by anion exchange chromatography on a 1-ml column of DEAE-Sephacel. After washing with 5-column volumes of 1% NaCl, bound material was eluted with 16% NaCl, dialyzed against water, and concentrated by vacuum centrifugation.

Results

NK1 in Hepatocytes and Mink Lung Cells

We found that although NK1 blocked the effects of HGF in hepatocytes, it was only a weak inhibitor of HGF in mink lung cells and had activity of its own in the absence of HGF (Fig. 1). This type of behavior is characteristic of a partial agonist, which weakly activates the receptor but occupies the binding site, blunting responses to a full agonist. The antagonistic activity of NK1 can be explained by its ability to competitively inhibit HGF binding to c-Met (16), but to have agonist and antagonist activity in different cell lines was surprising. Because (a) NK1 binds heparin (16);

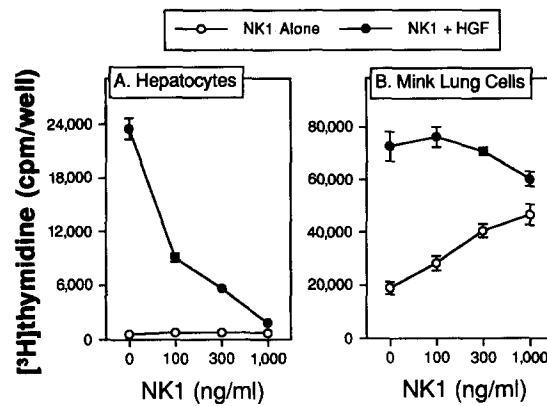


Figure 1. NK1 is antagonist in hepatocytes but partial agonist in mink lung cells. Hepatocytes (A) or mink lung cells (B) were cultured overnight with the indicated concentrations of NK1 alone or NK1 with a maximally effective concentration of HGF (30 ng/ml for hepatocytes, 10 ng/ml for mink lung cells). [^3H]thymidine was added, and 24 h later the amount incorporated into DNA was measured. Shown are the mean \pm SEM of six replicates from a representative experiment.

(b) heparin can augment the activity of HGF (41) and other heparin-binding growth factors (1, 26, 39); and (c) heparin-like molecules on the cell surface are important for FGF activity (13, 32), we hypothesized that the agonist activity of NK1 in mink lung cells might be due to local activation of NK1 by proteoglycans on the surface of mink lung cells. Consistent with that hypothesis, Table I shows that mink lung cells have a large capacity to bind NK1 and that most of the NK1 binding is displaced by heparin, suggesting it is to a heparin-like molecule.

Heparin Converts NK1 from Antagonist to Partial Agonist in Hepatocytes

One prediction from our hypothesis is that NK1 would become an agonist in the presence of the appropriate glycosaminoglycan. This was tested in hepatocytes, using heparin as the glycosaminoglycan. As shown in Fig. 2 A, there were two changes when heparin was added to the culture medium. First, the response to this dose of HGF (10 ng/ml) increased, which is consistent with our earlier report (41). Second, the response to NK1 switched from antagonist to partial agonist and resembled qualitatively the response

Table I. Heparin Displaces NK1 Binding to Mink Lung Cells

| NK1 | Heparin | Specific binding | Inhibition by heparin |
|-------|-------------------------|--------------------|-----------------------|
| ng/ml | $\mu\text{g}/\text{ml}$ | Fluorescence units | % |
| 0 | 0 | 0.0 | |
| 100 | 0 | 18.7 | |
| 100 | 1 | 0.4 | 97.9 |
| 1,000 | 0 | 130.2 | |
| 1,000 | 1 | 6.4 | 95.1 |

Mink lung cells were incubated with the indicated concentrations of NK1 in the absence and presence of 1 $\mu\text{g}/\text{ml}$ heparin for 1 h at 4°C. The cells were then washed twice with PBS, and the amount of NK1 bound was detected by adding an mAb to NK1 followed by phycoerythrin-streptavidin and quantitation by flow cytometry. Specific binding was calculated by subtracting the amount of fluorescence detected in the absence of NK1 (4.8 fluorescence units). Data are the mean of duplicates that varied <5%.

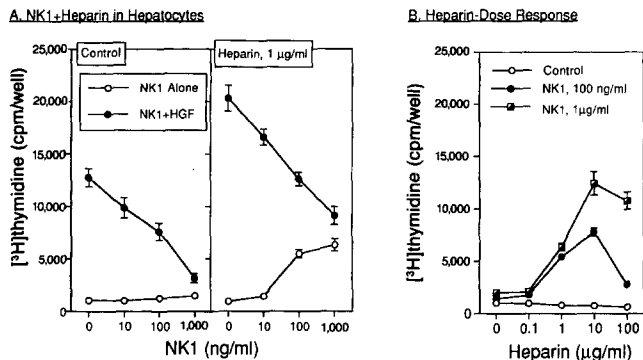


Figure 2. Heparin converts NK1 to agonist in hepatocytes. (A) Hepatocytes were cultured overnight in the presence of the indicated concentrations of NK1 alone or with 10 ng/ml HGF in either control medium (left) or medium containing 1 μg/ml heparin (right). [³H]thymidine was added, and 24 h later the amount incorporated into DNA was measured. (B) Hepatocytes were cultured for 24 h with 0.1 (closed circles) or 1 μg/ml (half-filled squares) NK1 plus the indicated concentrations of heparin. [³H]thymidine was added, and 24 h later the amount incorporated into DNA was measured. Data shown in A and B are the mean ± SEM of six replicates from a representative experiment.

to NK1 in mink lung cells (see Fig. 1 B). The heparin dose-response curve was bell-shaped, with maximal responses at ~10 μg/ml heparin (Fig. 2 B).

NK1 + Heparin Has Agonist Activity in BaF3 Cells Expressing c-Met

The synergy between NK1 and heparin was further investigated in BaF3 cells, which are reported to lack cell surface heparan sulfate proteoglycans (25). These cells do not normally express c-Met and are insensitive to HGF, but, as illustrated in Fig. 3 A, HGF was able to stimulate DNA synthesis in BaF3-hMet cells, which were derived by transfection with a cDNA for full-length human c-Met. Responses to HGF were observed in both the absence and presence of heparin, but heparin increased the sensitivity to HGF ~10-fold, with no effect on the magnitude of the maxi-

mal response. We recently reported a similar sensitizing effect of heparin on HGF responses in rat hepatocytes (41).

As in hepatocytes, NK1 stimulated DNA synthesis in BaF3-hMet cells when heparin was included in the medium (Fig. 3 B). The difference in potency between NK1 and HGF is consistent with the lower affinity of NK1 for c-Met (16). The effects of heparin on HGF and NK1 responsiveness were specific, as heparin had no effect on the response to IL-3 (Fig. 3 C). Moreover, there was no effect of HGF or NK1 with or without heparin in control BaF3-neo cells that had been transfected with an empty vector (Fig. 4). These data indicate that the response to NK1 + heparin is specific and is mediated through c-Met.

Consistent with the data in hepatocytes, the heparin dose-response curve in BaF3-hMet cells was again bell-shaped (Fig. 5). Maximal responses occurred at 1–10 μg/ml heparin with 1 μg/ml NK1. A small response to NK1 at 1 μg/ml was sometimes observed in the absence of heparin, so we extended the NK1 dose-response curve. A modest response to NK1 was noted at 10 μg/ml in the absence of heparin, which then returned to baseline at 100 μg/ml NK1 (data not shown). However, in the presence of heparin, responses occurred at NK1 doses as low as 100 ng/ml, which had no effect in the absence of heparin (Fig. 5). A small effect of heparin alone was also sometimes observed, especially at 10 μg/ml (see Fig. 5). The reason is unclear, but at 1 μg/ml, which was the dose used in most studies, this effect was inconsistent and nonspecific in that it also occurred in BaF3-neo cells (see Fig. 4). In contrast, the effect of NK1 + heparin was always much larger, was consistently observed in every experiment, and was only seen in cells expressing c-Met.

DNA synthesis is necessary but not sufficient for cellular proliferation. To determine whether NK1 + heparin could stimulate progression through the entire cell cycle, growth was measured by counting cells after 7 d. As shown in Table II, NK1 alone had very little effect, but NK1 + heparin increased the number of cells by ~17-fold. For comparison, 10 ng/ml HGF in the absence of heparin (~ED50; see Fig. 3) caused a sevenfold increase, and in the presence of heparin (~ED90), a 24-fold increase.

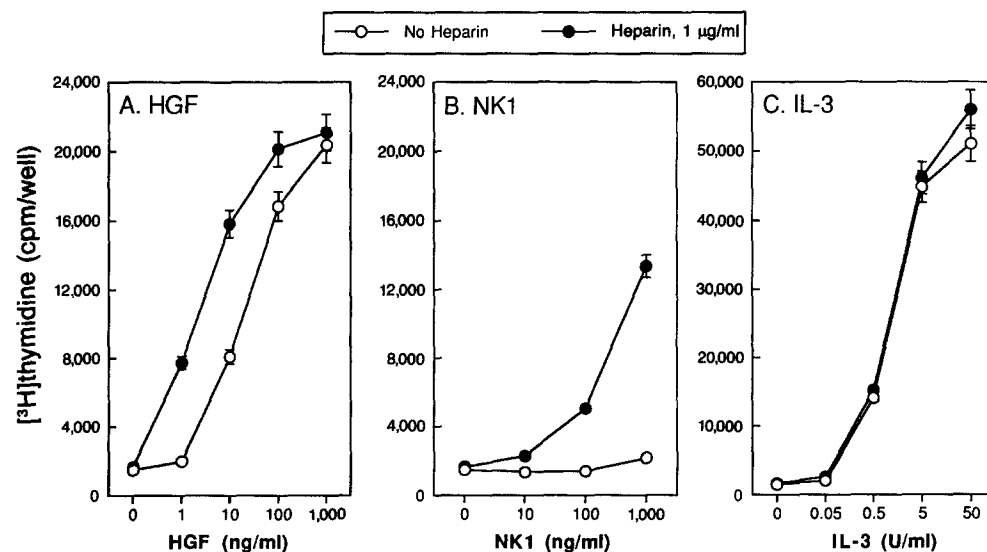


Figure 3. Heparin alters response of BaF3-hMet cells to HGF and NK1 but not to IL-3. BaF3-hMet cells were cultured 16 h with the indicated concentrations of HGF (A), NK1 (B), or IL-3 (C) in the absence (open circles) or presence (closed circles) of 1 μg/ml heparin. [³H]thymidine was added, and 7 h later the amount incorporated into DNA was measured. Data shown are the mean ± SEM of four replicates from a representative experiment.

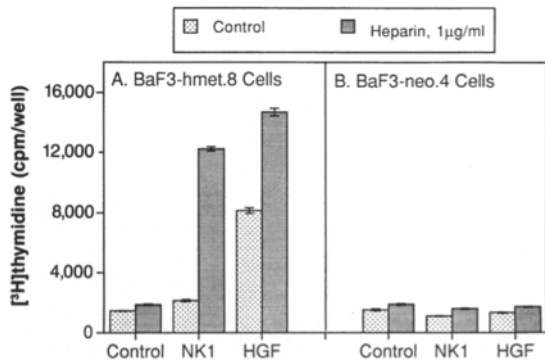


Figure 4. Heparin promotes NK1 activity in BaF3-hMet cells but not in BaF3-neo cells. BaF3-hMet cells (A), which were derived by transfection with a cDNA for full-length human c-Met, and BaF3-neo cells (B), transfected with the empty neo vector, were cultured for 16 h with 1 $\mu\text{g/ml}$ NK1 or 10 ng/ml HGF in the absence (hatched bars) or presence (gray bars) of 1 $\mu\text{g/ml}$ heparin. [^3H]thymidine was added, and 7 h later the amount incorporated into DNA was measured. Bars represent the mean \pm SEM of four replicates from a representative experiment.

Thus, NK1 + heparin is a true mitogen in these cells, capable of eliciting proliferative responses comparable to those to full-length HGF.

Heparin Specificity

To determine the specificity of the activating effect of heparin, a variety of sulfated and nonsulfated polysaccharides was tested. As shown in Table III, heparin, dextran sulfate, pentosan polysulfate, and chondroitin sulfate synergized with NK1, but de-N-sulfated heparin, dextran, and sodium sulfate had no effect. Heparan sulfate enhanced the response to full-length HGF but had no effect on NK1 activity, even at concentrations 100-fold greater than heparin. These data suggest that NK1 and HGF have different

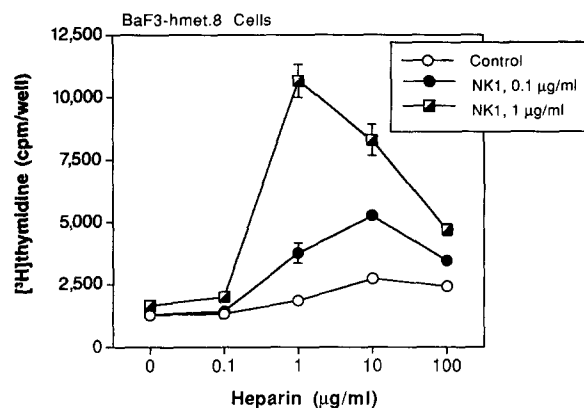


Figure 5. Heparin dose-response with NK1 in BaF3-hMet cells. BaF3-hMet cells were cultured for 16 h with 0.1 (closed circles) or 1 $\mu\text{g/ml}$ (half-filled squares) NK1 in the absence or presence of the indicated concentrations of heparin. [^3H]thymidine was added, and 7 h later the amount incorporated into DNA was measured. Shown are the mean \pm SEM of four replicates from a representative experiment.

Table II. NK1 + Heparin Stimulates Growth of BaF3-hMet Cells

| Addition | Cells per ml |
|-------------------------------------|----------------------|
| Control | 11,400 \pm 2,900 |
| NK1 (1 $\mu\text{g/ml}$) | 21,340 \pm 3,000 |
| NK1 + heparin (1 $\mu\text{g/ml}$) | 177,900 \pm 10,270 |
| HGF (10 ng/ml) | 76,700 \pm 5,400 |
| HGF + heparin (1 $\mu\text{g/ml}$) | 248,300 \pm 22,100 |

BaF3-hMet cells were seeded at a density of 26,000 cells per ml in medium containing 1 $\mu\text{g/ml}$ NK1 or 10 ng/ml HGF in the absence or presence of 1 $\mu\text{g/ml}$ heparin. After 7 d, the number of cells was counted. Data are the mean \pm SEM of four replicates from a representative experiment.

structural requirements for their interactions with sulfated polysaccharides.

Heparin Enhances NK1 Binding to BaF3-hMet Cells

A series of experiments was conducted to better understand the mechanism for the heparin-induced activation of NK1. One possibility is that heparin acts on the cells to induce responsiveness to NK1. However, BaF3-hMet cells that had been preincubated overnight with heparin did not respond when subsequently challenged with NK1 in the absence of heparin (data not shown). Thus, there is a requirement for both agents to be present simultaneously.

To determine whether heparin increased the cellular binding of NK1, BaF3-hMet cells were incubated with increasing concentrations of NK1, with and without heparin, and bound NK1 was detected with an excess of biotinylated anti-NK1 mAb, followed by phycoerythrin-streptavidin and fluorescence quantitation by flow cytometry. This approach avoids labeling-induced chemical alterations in the ligand, and although absolute amounts of NK1 bound cannot be determined, it is possible to measure affinity and to make relative comparisons of receptor number. Using this method, specific binding of NK1 was detected in BaF3-hMet cells, and the amount of binding was increased in the presence of heparin, whereas no binding of NK1 was detected in BaF3-neo cells in either presence or absence of heparin (Fig. 6 A). The binding of NK1 to BaF3-hMet cells was further characterized by performing Scatchard analysis of saturation binding curves. As shown in Fig. 6 B, heparin caused the apparent affinity of NK1 to increase \sim 10-fold, and the apparent number of receptors increased nearly twofold.

Heparin Promotes Stabilization and Dimerization of NK1

In the absence of heparin, NK1 did not antagonize HGF activity in BaF3-hMet cells (data not shown) at the same concentrations that inhibit binding of HGF to c-Met in cell-free systems (16) and blocked HGF activity in hepatocytes (Fig. 1). This was true whether the BaF3-hMet cells were tested in BaF3 medium or in hepatocyte medium (data not shown). To determine whether NK1 was being inactivated, NK1 was preincubated overnight at 37°C in BaF3 culture medium with or without heparin. Heparin was then added to samples that had been preincubated in the absence of heparin, and the ability of the preincubated samples to stimulate DNA synthesis in BaF3-hMet cells was

Table III. Effect of Sulfated and Nonsulfated Polysaccharides on the Activity of NK1, HGF, and NK2 in BaF3-hmet Cells

| CoFactor | Concentration $\mu\text{g/ml}$ | ^3H thymidine incorporation | | | ^3H thymidine incorporation | |
|----------------------|-----------------------------------|--------------------------------------|---------------------------|--------------------|--------------------------------------|--------------------|
| | | Control | NK1 (1 $\mu\text{g/ml}$) | HGF (10 ng/ml) | Control | NK2 (100 ng/ml) |
| | | <i>cpm per well</i> | | | <i>cpm per well</i> | |
| Control | — | 2,135 \pm 40 | 4,216 \pm 174 | 11,035 \pm 170 | 3,831 \pm 523 | 4,153 \pm 450 |
| Heparin | 10 | 3,323 \pm 67 | 12,558 \pm 145 | 15,482 \pm 358 | 6,016 \pm 260 | 9,674 \pm 514 |
| DE-N-Sulfate Heparin | 10 | 2,196 \pm 70 | 4,404 \pm 175 | 11,030 \pm 374 | 4,059 \pm 227 | 4,233 \pm 353 |
| Dextran sulfate | 10 | 3,638 \pm 60 | 11,013 \pm 340 | 17,119 \pm 335 | 6,101 \pm 89 | 11,583 \pm 1,015 |
| Dextran | 10 | 2,254 \pm 108 | 4,264 \pm 410 | 11,154 \pm 516 | 4,653 \pm 237 | 5,780 \pm 282 |
| Sodium sulfate | 10 | 2,374 \pm 93 | 4,690 \pm 65 | 11,043 \pm 467 | 6,049 \pm 484 | 5,204 \pm 227 |
| Pantosan polysulfate | 10 | 2,858 \pm 43 | 8,909 \pm 481 | 15,788 \pm 731 | 5,456 \pm 171 | 12,754 \pm 564 |
| Heparin sulfate | 10 | 2,021 \pm 72 | 5,330 \pm 197 | 13,989 \pm 559 | 4,131 \pm 195 | 7,266 \pm 648 |
| Control | — | 2,243 \pm 38 | 2,762 \pm 268 | 11,003 \pm 459 | 3,272 \pm 64 | 3,098 \pm 71 |
| Heparan | 1 | 2,547 \pm 116 | 6,668 \pm 366 | 18,640 \pm 393 | 3,262 \pm 133 | 6,916 \pm 202 |
| Heparan sulfate | 1 | 2,083 \pm 110 | 2,446 \pm 75 | 16,718 \pm 595 | ND | ND |
| | 10 | 1,912 \pm 133 | 2,641 \pm 77 | 16,732 \pm 750 | 2,554 \pm 94 | 4,602 \pm 210 |
| | 100 | 1,996 \pm 132 | 3,197 \pm 116 | 13,538 \pm 1,021 | ND | ND |
| Control | — | 2,918 \pm 142 | 2,792 \pm 202 | ND | 2,918 \pm 142 | 2,472 \pm 90 |
| Heparin | 1 | 2,112 \pm 119 | 6,758 \pm 212 | ND | 2,112 \pm 119 | 6,247 \pm 178 |
| Chondroitin sulfate | 100 | 2,460 \pm 99 | 5,039 \pm 230 | ND | 2,460 \pm 99 | 5,042 \pm 47 |

BaF3-hmet cells were cultured for 16 h with NK1, HGF, or NK2 in combination with a variety of sulfated and nonsulfated polysaccharides. ^3H thymidine was added and the amount incorporated into DNA was measured 7 h later. Values shown are the mean \pm SEM of four replicates from a representative experiment.

A. NK1 Binding to BaF3-hMet vs BaF3-neo Cells

| Addition | NK1 Bound (Fluorescence Units) | |
|-------------|-----------------------------------|----------|
| | BaF3-hMet | BaF3-neo |
| None | 0.00 | 0.00 |
| Heparin | 0.01 | 0.00 |
| NK1 | 1.02 | 0.02 |
| NK1+Heparin | 2.67 | 0.03 |

B. Scatchard Analysis

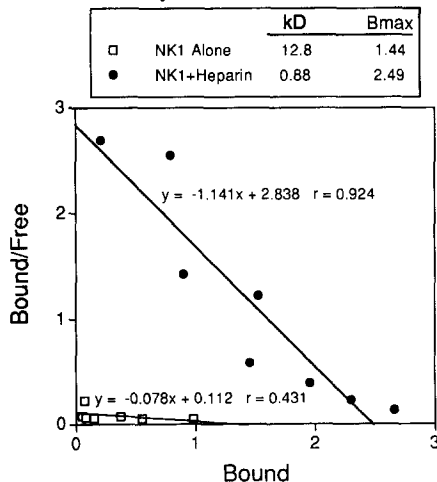


Figure 6. Heparin enhances binding of NK1 to BaF3-hMet cells. (A) BaF3-hMet cells and BaF3-neo cells were incubated for 1 h at 4°C with 10 nM NK1 in the absence or presence of 1 $\mu\text{g/ml}$ heparin. Unbound NK1 was washed away, and bound NK1 was detected with biotinylated anti-NK1 mAb 6D5 followed by phycoerythrin-streptavidin and quantitation by flow cytometry. 10,000 cells were analyzed, and the mean fluorescent intensity was calculated. Nonspecific fluorescence in the absence of NK1 has been subtracted. Shown are the means of duplicates that did not differ by >8%. (B) Scatchard analysis was performed on saturation binding data for NK1 binding to BaF3-hMet cells in the absence (open squares) and presence (closed circles) of 1 $\mu\text{g/ml}$ heparin. Each point is the mean of duplicates that varied <10%.

measured, compared to freshly prepared NK1 or NK1 + heparin. As shown in Table IV, NK1 that had been preincubated overnight in medium without heparin was completely inactive, even though heparin was present when the cells were challenged with the sample. In contrast, NK1 that was preincubated with heparin retained some activity.

Receptor dimerization is an important process in growth factor receptor activation, and the bell shape of the heparin dose-response curve is consistent with a dimerization mechanism. For these reasons, we examined the ability of heparin to induce dimerization of NK1. A bifunctional cross-linker was added to solutions of NK1 alone or NK1 with heparin. The samples were then separated by SDS-PAGE, and bands containing NK1 were identified by Western blotting. The results are shown in Fig. 7. In the absence of heparin, a single band with apparent molecular weight of \sim 26,000 was detected. In the presence of heparin, there was an additional band at \sim 53,000, consistent with an NK1 dimer, along with smaller amounts of a larger species consistent with a trimer. The larger molecular

Table IV. Heparin Stabilizes NK1 in BaF3 Medium

| Addition | ^3H thymidine incorporation <i>cpm per well</i> |
|----------------------------------|---|
| Control | 4,566 \pm 84 |
| NK1 | 4,867 \pm 156 |
| Heparin | 5,222 \pm 176 |
| NK1 + heparin | 12,312 \pm 139 |
| NK1 preincubated without heparin | 5,285 \pm 201 |
| NK1 preincubated with heparin | 7,862 \pm 84 |

NK1 (100 ng/ml) was preincubated with or without 1 $\mu\text{g/ml}$ heparin in BaF3 culture medium for 16 h at 37°C, 5% CO₂. The next morning BaF3-hMet cells were added. At this time, heparin was also added to the NK1 that was preincubated without heparin so that heparin would be present during the challenge incubation. For comparison, cells were also added to wells containing freshly prepared NK1 and heparin. The next morning ^3H thymidine was added, and the amount incorporated into DNA was measured 7 h later. Shown are the mean \pm SEM of four replicates from a representative experiment.

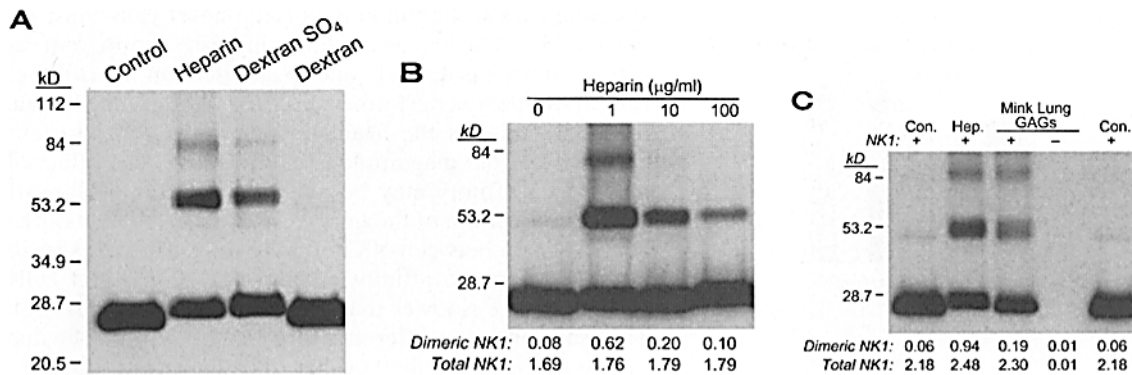


Figure 7. Dimerization of NK1. (A) The bifunctional cross-linker BS³ was added to tubes containing NK1 alone or NK1 with heparin, dextran sulfate, or dextran. The samples were then analyzed by Western blotting with anti-NK1 antibodies. Molecular weight markers are shown on the side. (B) Samples of NK1 with increasing concentrations of heparin were analyzed as described above. (C) NK1 was incubated with a crude preparation of glycosaminoglycans from mink lung cells (see Materials and Methods) and analyzed as described for A. The numbers under the lanes in B and C are OD readings from the dimeric NK1 and total NK1 ELISAs.

weight bands were also observed with dextran sulfate, which activates NK1, but not with dextran, which does not activate NK1 (see Table III). The higher molecular weight bands were not detected when the cross-linker was omitted (data not shown).

To confirm that the higher molecular weight bands were oligomers, we adapted the NK1 ELISA to detect dimeric and multimeric, but not monomeric, NK1. By using a single anti-NK1 mAb for both capture and detection, monomeric NK1 is not detected because binding of the epitope by the capture antibody prevents its binding to the detection antibody. Dimeric NK1, however, presents an additional epitope for the detection antibody. In the dimer format, a signal was detected for NK1 with heparin but not for either reagent alone (Table V). In the normal format, OD was detected for NK1 alone and for NK1 plus heparin, indicating that NK1 was present in both samples. The greater OD for NK1 plus heparin vs NK1 alone in the normal format is also consistent with dimerization/multimerization, as additional epitopes for the detection antibody would be present on each captured molecule.

The heparin dose-response curves for NK1 activation in both hepatocytes and BaF3-hMet cells were bell-shaped (see Figs. 2 B and 5); i.e., the response diminished at high concentrations of heparin. As shown in Fig. 7 B, dimerization of NK1 was also diminished as the concentration of heparin was increased. Interestingly, a preparation of GAGs from the surface of mink lung cells was also able to induce dimerization of NK1 (Fig. 7 C). A faint band in the

dimer position was sometimes observed for NK1 samples without heparin, suggesting that NK1 may have a natural but weak tendency to dimerize.

Heparin Promotes Agonist Activity of Naturally Occurring Variant NK2

All of the experiments shown above were conducted with NK1, but similar results have also been obtained with the naturally occurring variant NK2, which also binds heparin. As shown in Fig. 8 A, NK2 had activity in mink lung cells, and the response was similar to that induced by NK1 and by HGF. Fig. 8 A also shows that the response of mink lung cells to NK1, NK2, and HGF is not affected by heparin. In contrast, in BaF3-hMet cells NK2, like NK1, had no activity in the absence of heparin but stimulated DNA synthesis when heparin was present (Fig. 8 B). Although the absolute magnitude of the response to NK2 + heparin was only approximately twofold in this particular experiment, it was similar to that induced by NK1 + heparin and by HGF. Importantly, there was no stimulatory effect of NK2 + heparin in BaF3-neo cells (Fig. 8 C), indicating that the effect of NK2 + heparin is specific and, like the effect of NK1 + heparin and HGF, is mediated through c-Met. As with NK1, the effect of heparin on NK2 activity in BaF3-hMet cells was mimicked by dextran sulfate, pentosan polysulfate, and chondroitin sulfate, but not by de-N-sulfated heparin, dextran, or sodium sulfate (Table III). Interestingly, heparan sulfate activated NK2, although it had no effect on NK1.

Table V. Heparin Induces NK1 Dimerization

| Sample | NK1 ELISA (OD 492-405) | |
|---------------|------------------------|---------------|
| | Dimer format | Normal format |
| Control | 0.004 ± 0.000 | 0.004 ± 0.001 |
| NK1 | 0.007 ± 0.000 | 0.875 ± 0.018 |
| Heparin | 0.006 ± 0.000 | 0.003 ± 0.000 |
| NK1 + heparin | 0.323 ± 0.025 | 2.620 ± 0.012 |

NK1 that had been incubated in the presence or absence of heparin was cross-linked with BS³ and then assayed in the normal NK1 ELISA, which recognizes monomeric and dimeric NK1 (right column) or an adaptation of the ELISA designed to detect only dimeric NK1 (left column). Shown are OD values after development of the HRP. Data are the mean ± SEM of four replicates.

Discussion

We show here that the truncated HGF variant NK1, which is an HGF antagonist in hepatocytes, acts as a partial agonist in mink lung cells, and interestingly, that heparin can convert NK1 from an antagonist to a partial agonist in hepatocytes. Heparin also conferred agonist activity onto NK1 in c-Met transfected BaF3 cells, which do not express the proteoglycan core protein syndecan and do not have endogenous cell surface proteoglycans (25). That the response in BaF3-hMet cells was specific was supported by the findings that heparin had no effect on IL-3 sensitivity in

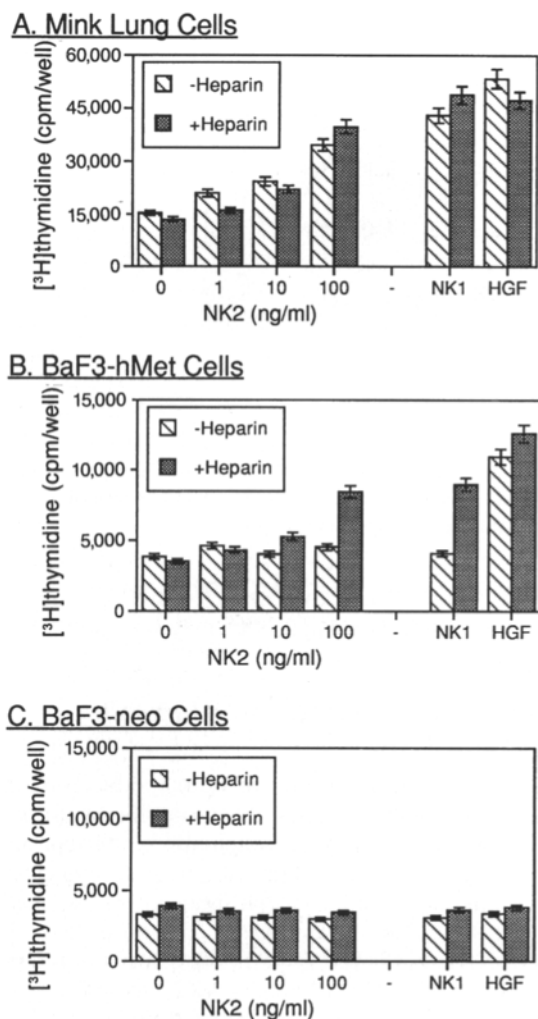


Figure 8. NK2 has proliferative activity on mink lung cells and BaF3-hMet cells. Mink lung cells (A), BaF3-hMet cells (B), and BaF3-neo cells (C) were cultured with the indicated concentrations of NK2, in the absence or presence of 1 μ g/ml heparin. [³H]thymidine incorporation into DNA was measured. As positive controls, 100 ng/ml NK1 and 10 ng/ml HGF were also included. Bars in each panel represent the mean \pm SEM of four replicates from a representative experiment.

these cells and that NK1 + heparin had no effect on BaF3-neo cells. The latter observation also indicates that the effects of NK1 + heparin is mediated through c-Met, the same receptor that mediates responses to full-length HGF.

The concentrations over which NK1 was an agonist in mink lung cells and over which NK1 + heparin was an agonist in hepatocytes and BaF3-hMet cells were high compared to those of HGF. This is due in part to the lower affinity of NK1 for c-Met (16), combined with the lower efficiency for activating c-Met, as reflected in partial agonism. Nonetheless, the concentrations of NK1 in which we observed agonist activity in our studies are similar to those in which we (Figs. 1 and 2) and others (16) have observed antagonistic activity in hepatocytes.

Mechanistic studies revealed that heparin enhanced the binding of BaF3-hMet cells, and Scatchard analysis revealed that both the apparent affinity and receptor number were increased. Since there was no binding of NK1 to

BaF3-neo cells, the binding to BaF3-hMet cells must involve c-Met. The heparin-induced increase in apparent receptor number is unlikely due to an effect on the cells because binding was performed at 4°C; rather, it may be due to dimerization of the ligand, which would be consistent with the twofold magnitude of the increase. The effect of heparin on affinity may be attributed to stabilization of NK1, dimerization of the ligand, or a bridging effect of the heparin chain between NK1 and the cell surface. It should be noted that the affinity of NK1 for BaF3-hMet cells measured here is lower than that reported for c-Met (16). The reason for this difference is unclear, but it may be due to differences in the methods used to measure binding.

That the heparin dose-response curve for NK1 activity was bell-shaped is consistent with a dimerization mechanism; as the concentration of the dimerizing agent increases, the ratio of it to the dimerized factor goes from 1:2 to 1:1. Direct evidence for a role of dimerization came from cross-linking studies that revealed that heparin induced NK1 to form dimers, along with smaller amounts of higher order multimers. Dextran sulfate, which activates NK1 bioactivity, also induced dimerization, whereas similarly sized but unsulfated dextran, which has no effect on NK1 bioactivity, did not. Moreover, we found that the heparin dose-response curve for dimerization, like the heparin dose-response curve for NK1 activity, was bell-shaped. A very faint band in the dimer position was sometimes observed in samples of NK1 without heparin, suggesting that NK1 may have a tendency to form weakly associated dimers spontaneously. Heparin and related glycosaminoglycans may stabilize the dimer, shifting the equilibrium toward the dimer. Taken together, the cross-linking data suggest that dimerization is important in the heparin mechanism.

Based on our findings with exogenous heparin, we propose that the agonist activity of NK1 in mink lung cells is due to an activating interaction of NK1 with endogenous heparin-like glycosaminoglycans on the cell surface. Consistent with that hypothesis, we found that mink lung cells had a high capacity to bind NK1 and that most of the NK1 binding could be inhibited by heparin. The inhibitory effect of heparin on NK1 binding was not a general phenomenon because it had the opposite effect, i.e., increased NK1 binding, in BaF3-hMet cells, which do not express cell surface HSPGs. Thus, in mink lung cells NK1 interacts with a heparin-like molecule on the surface. The nature of this hypothetical cofactor is unknown, but we found that a variety of sulfated polysaccharides could substitute for heparin. Sulfation was critical for activity, but the sulfate moiety had to be present in the context of a polysaccharide backbone. Heparan sulfate had no effect on NK1 activity but enhanced the response to NK2 and full-length HGF, suggesting that heparan sulfate may interact selectively with kringle 2, which has been identified as a heparin-binding region in full-length HGF (19). The negative data with heparan sulfate and NK1 also suggests that if our model is correct, then glycosaminoglycans on mink lung cells must resemble heparin more than heparan sulfate. Alternatively, commercial preparations of heparan sulfate may differ in charge and charge-density from heparan sulfate on the surface of a living mink lung cell.

We made several attempts to determine whether the response of mink lung cells to NK1 could be abrogated by di-

gesting the endogenous heparan sulfate proteoglycans with heparatinase. Although such treatment removed ~80% of the heparin-displaceable NK1 binding, the cells still responded to NK1 (data not shown). Adding chlorate to the medium also failed to block NK1 responses (data not shown). There are several potential explanations for these observations. First, the proposed NK1 cofactor could be insensitive to heparatinase and turn over too slowly for its sulfation to be affected by chlorate in the time frame of our experiments. Consistent with that concept, heparinase treatment completely eliminated heparin-displaceable HGF binding (data not shown), but a residual amount of heparin-displaceable NK1 binding always remained. Moreover, as noted above, our finding that heparan sulfate could not substitute for heparin suggests that the proposed NK1 cofactor is more similar to heparin than heparan sulfate. That would also be consistent with our heparatinase data because heparatinase cleaves heparan sulfate-like glycosaminoglycans but not heparin-like glycosaminoglycans. Alternatively, the endogenous cofactor that we have hypothesized could be chondroitin sulfate or another sulfated glycosaminoglycan. In addition, it could be a sulfoglycolipid such as galactosylceramide 3'-sulfate, which has been shown to bind HGF with high affinity (14). Interestingly, the binding of HGF to this sulfated lipid is competed by heparin (14).

Although we have been unable to nullify NK1 activity by enzymatically removing endogenous glycosaminoglycans, a crude preparation of cell surface proteoglycans from mink lung cells induced dimerization of NK1. The principle responsible for the dimerization activity in this preparation is undefined, but it is resistant to trypsin and boiling and has sufficient negative charge at neutral pH to bind tightly to an anion exchange resin. These characteristics are consistent with a glycosaminoglycan. That the dimerizing activity was released by trypsin suggests that it was derived from a proteoglycan, providing strong evidence to support our model. The nature of the active component in this preparation is currently under evaluation.

The stabilizing effect of heparin on NK1 may be important in interpreting some of the data from BaF3-hMet cells. As noted above, a small [³H]thymidine response in BaF3-hMet cells was observed with high doses of NK1 in the absence of heparin. Combined with the decay of bioactive NK1 in BaF3 medium without heparin, this result suggests that the normal response to NK1 in BaF3-hMet cells may be agonistic, but that the response is weak because NK1 is unstable. This would also explain why NK1 in the absence of heparin was a poor HGF antagonist in these cells. If our model is correct, then BaF3 cells may have low amounts of an endogenous factor that can activate NK1. Although BaF3 cells do not express the proteoglycan core protein syndecan (25), there is a report that 32D cells, which resemble BaF3 in many ways, incorporate ³²SO₄ into a 150-kD protein that is resistant to heparinase but sensitive to chondroitinase (27), and we have found that chondroitin sulfate activates NK1 (see Table III). Other candidates that could subserve this function include glycosaminoglycans tethered to the cell membrane through phosphoinositol linkages (12), and the above noted sulfoglycolipids, which have been reported to bind tightly to HGF (14).

Although the synergism between heparin and NK1 in

BaF3-hMet cells may be due, at least partly, to stabilization of NK1, other mechanisms must also be involved. In hepatocytes, NK1 is an antagonist in the absence of heparin, indicating that it is stable under those conditions but behaves as an antagonist rather than an agonist. One possibility is that hepatocytes have an endogenous inhibitor that prevents NK1 from activating c-Met. However, the fact that heparin converts NK1 to an agonist in hepatocytes, and does so at heparin concentrations similar to those that work in BaF3-hMet cells, argues against an inhibitor. Our cross-linking studies strongly argue that the other mechanism is heparin-induced dimerization, which in turn can facilitate dimerization and activation of c-Met. We propose that the endogenous glycosaminoglycans on hepatocytes are incompetent in this regard, but responses can be elicited if exogenous heparin is supplied. By analogy, the glycosaminoglycans of B5/589 cells, in which NK2 is an antagonist (3), must also be incompetent. How such competence is conferred is unclear, but our results suggest that the degree of sulfation may be one mechanism.

We have shown that heparin and heparin-like glycosaminoglycans can also confer proliferative activity on the naturally occurring variant NK2, which, like NK1, was originally described as a competitive HGF antagonist (3). Since NK2 is very similar to NK1, most of the mechanistic discussion above should be equally applicable to NK2, with the exception that heparan sulfate was able to promote the activity of NK2 but not NK1. The latter observation suggests that heparan sulfate may interact with kringle 2, which has been identified as a heparin-binding domain in full-length HGF (19). Because NK2 mRNA is expressed in normal tissues such as the placenta (18), it is likely to have a physiological function in regulating HGF sensitivity. Our data suggest that glycosaminoglycans can be important in determining whether NK2 antagonizes HGF activity, providing a novel mechanism by which net HGF activity might be fine tuned.

Our findings might also help to explain why NK2 retains the scattering but not the mitogenic activity of full-length HGF (10). Those authors concluded that binding to c-Met by NK2 selectively triggers the intracellular signals involved in the motility response, but that full-length HGF is required to trigger intracellular signaling cascade involved in mitogenesis. However, scattering was analyzed in MDCK cells, while mitogenesis was tested in hepatocytes. The agonist activity of NK2 on MDCK cells could be analogous to the agonist activity of NK1 and NK2 we observed in mink lung cells; i.e., NK1 and NK2 may be mimicking a normal HGF response in each cell type: scattering in MDCK cells and DNA synthesis in mink lung cells. Thus, the selective scattering activity of NK2 may be related more to differences in the proteoglycans on the surface of the cells used to test scattering and mitogenesis than on the ability of NK2 to preferentially activate a scattering domain of c-Met.

In conclusion, we report here that heparin and heparin-like glycosaminoglycans synergize with NK1 and the naturally occurring variant NK2 to promote proliferative activity in cells expressing the HGF receptor, c-Met. Clearly, NK1 + heparin is not equivalent to full-length HGF, and regions of HGF from kringle 1 to the COOH terminus are very important in HGF's potency and biological activity.

But the ability of heparin and related glycosaminoglycans to confer any agonistic activity onto NK1 and NK2 has implications for the use of these variants to pharmacologically antagonize HGF. In addition, our results suggest that changes in glycosaminoglycan structure on the surface of a target cell, either through changes in proteoglycan expression or through alterations inflicted by enzymes in the milieu, may provide a novel mechanism by which responsiveness to endogenous NK2, and thereby to HGF, can be locally regulated in different physiological and developmental states.

We thank Louise Richardson for technical assistance and investigations into NK1 dimerization, Michele Butler for help in the purification of NK1 and NK2, and the staff of the Flow Cytometry Laboratory for technical assistance. We also thank Greg Bennett for his help in developing the NK1/NK2 ELISA. We acknowledge Dr. Robert Linhardt for his advice on the preparation of glycosaminoglycans, and Dr. Robert Cohen for helpful discussion.

Received for publication 14 September 1995 and in revised form 26 January 1996.

References

- Aviezer, D., and A. Yayon. 1994. Heparin-dependent binding and autophosphorylation of epidermal growth factor (EGF) receptor by heparin-binding EGF-like growth factor but not by EGF. *Proc. Natl. Acad. Sci. USA* 91:12173-12177.
- Bottaro, D.P., J.S. Rubin, D.L. Faletto, A.M. Chan, T.E. Kmieciak, G.F. Vande Woude, and S.A. Aaronson. 1991. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science (Wash. DC)* 251:802-804.
- Chan, A.M., J.S. Rubin, D.P. Bottaro, D.W. Hirschfield, M. Chedid, and S.A. Aaronson. 1991. Identification of a competitive HGF antagonist encoded by an alternative transcript. *Science (Wash. DC)* 254:1382-1385.
- Di Renzo, M.F., R.P. Narsimhan, M. Olivero, S. Bretti, S. Giordano, E. Medico, P. Gaglia, P. Zara, and P.M. Comoglio. 1991. Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene* 6:1997-2003.
- Di Renzo, M.F., M. Olivero, G. Serini, F. Orlandi, S. Pilotti, A. Belfiore, A. Costantino, R. Vigneri, A. Angeli, M.A. Pierotti et al. 1995. Overexpression of the c-MET/HGF receptor in human thyroid carcinomas derived from the follicular epithelium. *J. Endocrinol. Invest.* 18:134-139.
- Ebert, M., M. Yokoyama, H. Friess, M.W. Büchler, and M. Korc. 1994. Co-expression of the c-met proto-oncogene and hepatocyte growth factor in human pancreatic cancer. *Cancer Res.* 54:5775-5778.
- Garrison, J.C., and R.C. Haynes, Jr. 1975. The hormonal control of gluconeogenesis by regulation of mitochondrial pyruvate carboxylation in isolated rat liver cells. *J. Biol. Chem.* 250:2769-2777.
- Gherardi, E., and M. Stoker. 1991. Hepatocyte growth factor-Scatter factor: mitogen, motogen and met. *Cancer Cells A Mon. Rev.* 3:227-232.
- Giordano, S., M.F. Di Renzo, R. Narsimhan, C.S. Cooper, C. Rosa, and P. Comoglio. 1989. Biosynthesis of the protein encoded by the c-met proto-oncogene. *Oncogene* 4:1383-1388.
- Hartmann, G., L. Naldini, K.M. Weidner, M. Sachs, E. Vigna, P.M. Comoglio, and W. Birchmeier. 1992. 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. USA* 89:11574-11578.
- Higashio, K., N. Shima, M. Goto, Y. Itagaki, M. Nagao, H. Yasuda, and T. Morinaga. 1990. Identity of a tumor cytotoxic factor from human fibroblasts and hepatocyte growth factor. *Biochem. Biophys. Res. Commun.* 170:397-404.
- Jackson, R.L., S.J. Busch, and A.D. Cardin. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.* 71:481-539.
- Klagsbrun, J., and A. Baird. 1991. A dual receptor system is required for basic fibroblast growth factor activity. *Cell* 67:229-231.
- Kobayashi, T., K. Honke, T. Miyazaki, K. Matsumoto, T. Nakamura, I. Ishizuka, and A. Makita. 1994. Hepatocyte growth factor specifically binds to sulfoglycolipids. *J. Biol. Chem.* 269:9817-9821.
- Kuniyasu, H., W. Yasui, H. Yokozaki, Y. Kitadai, and E. Tahara. 1993. Aberrant expression of c-met mRNA in human gastric carcinomas. *Int. J. Cancer* 55:72-75.
- Lokker, N.A., and P.J. Godowski. 1993. Generation and characterization of a competitive antagonist of human hepatocyte growth factor, HGF/NK1. *J. Biol. Chem.* 268:17145-17150.
- Matsumoto, K., and T. Nakamura. 1992. Hepatocyte growth factor: molecular structure, roles in liver regeneration, and other biological functions. *Crit. Rev. Oncog.* 3:27-54.
- Miyazawa, K., A. Kitamura, D. Naka, and N. Kitamura. 1991. An alternatively processed mRNA generated from human hepatocyte growth factor gene. *Eur. J. Biochem.* 197:15-22.
- Mizuno, K., H. Inoue, M. Hagiya, S. Shimizu, T. Nose, Y. Shimohigashi, and T. Nakamura. 1994. Hairpin loop and second kringle domain are essential sites for heparin binding and biological activity of hepatocyte growth factor. *J. Biol. Chem.* 269:1131-1136.
- Montesano, R., K. Matsumoto, T. Nakamura, and L. Orci. 1991. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 67:901-908.
- Nakamura, T. 1991. Structure and function of hepatocyte growth factor. *Prog. Growth Factor Res.* 3:67-86.
- Naldini, L., E. Vigna, R.P. Narsimhan, G. Gaudino, R. Zarnegar, G.K. Michalopoulos, and P.M. Comoglio. 1991. Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. *Oncogene* 6:501-504.
- Naldini, L., K.M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Ponzetto, R.P. Narsimhan, G. Hartmann, R. Zarnegar, G.K. Michalopoulos et al. 1991. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2867-2878.
- Natali, P.G., M.R. Nicotra, M.F. Di Renzo, M. Prat, A. Bigotti, R. Cavaliere, and P.M. Comoglio. 1993. Expression of the c-Met/HGF receptor in human melanocytic neoplasms: demonstration of the relationship to malignant melanoma tumour progression. *Br. J. Cancer.* 68:746-750.
- Ornitz, D.M., A. Yayon, J.G. Flanagan, C.M. Svahn, E. Levi, and P. Leder. 1992. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell Biol.* 12:240-247.
- Rapraeger, A.C., A. Krufka, and B.B. Olwin. 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science (Wash. DC)* 252:1705-1708.
- Roghani, M., A. Mansukhani, P. Dell'Era, P. Bellosta, C. Basilio, D.B. Rifkin, and D. Moscatelli. 1994. Heparin increases the affinity of basic fibroblast growth factor for its receptor but is not required for binding. *J. Biol. Chem.* 269:3976-3984.
- Rong, S., M. Bodescot, D. Blair, J. Dunn, T. Nakamura, K. Mizuno, M. Park, A. Chan, S. Aaronson, and G.F. Vande Woude. 1992. Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. *Mol. Cell Biol.* 12:5152-5158.
- Rong, S., M. Jeffers, J.H. Resau, I. Tsarfaty, M. Oskarsson, and G.F. Vande Woude. 1993. Met expression and sarcoma tumorigenicity. *Cancer Res.* 53:5355-5360.
- Rong, S., L.A. Donehower, M.F. Hansen, L. Strong, M. Tainsky, M. Jeffers, J.H. Resau, E. Hudson, I. Tsarfaty, and G.F. Vande Woude. 1995. Met proto-oncogene product is overexpressed in tumors of p53-deficient mice and tumors of Li-Fraumeni patients. *Cancer Res.* 55:1963-1970.
- Rosen, E.M., S.K. Nigam, and I.D. Goldberg. 1994. Scatter factor and the c-met receptor: a paradigm for mesenchymal/epithelial interaction. *J. Cell Biol.* 127:1783-1787.
- Rouslahti, E., and Y. Yamaguchi. 1991. Proteoglycans as modulators of growth factor activities. *Cell* 64:867-869.
- Sasaki, M., M. Nishio, T. Sasaki, and J. Enami. 1994. Identification of mouse mammary fibroblast-derived mammary growth factor as hepatocyte growth factor. *Biochem. Biophys. Res. Commun.* 199:772-779.
- Sonnenberg, E., D. Meyer, K.M. Weidner, and C. Birchmeier. 1993. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J. Cell Biol.* 123:223-235.
- Stoker, M. 1989. Effect of scatter factor on motility of epithelial cells and fibroblasts. *J. Cell. Physiol.* 139:565-569.
- Weidner, K.M., J. Behrens, J. Vandekerckhove, and W. Birchmeier. 1990. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J. Cell Biol.* 111:2097-2108.
- Weidner, K.M., M. Sachs, and W. Birchmeier. 1993. The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J. Cell Biol.* 121:145-154.
- Yamashita, J.-i., M. Ogawa, S.-i. Yamashita, K. Nomura, M. Kuramoto, T. Saishoji, and S. Shin. 1994. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. *Cancer Res.* 54:1630-1633.
- Yayon, A., M. Klagsburg, J.D. Esko, P. Leder, and D.M. Ornitz. 1991. Cell surface heparin-like molecules are required for binding of bFGF to its high affinity receptor. *Cell* 64:841-848.
- Yoshinaga, Y., Y. Matsuno, S. Fujita, T. Nakamura, M. Kikuchi, Y. Shimamoto, and S. Hirohashi. 1993. Immunohistochemical detection of hepatocyte growth factor/scatter factor in human cancerous and inflammatory lesions of various organs. *Jpn. J. Cancer Res.* 84:1150-1158.
- Zioncheck, T.F., L. Richardson, J. Liu, L. Chang, K.L. King, G. L. Bennett, P. Fugedi, S.M. Chamow, R.H. Schwall, and R.J. Stack. 1995. Sulfated oligosaccharides promote hepatocyte growth factor association and govern its mitogenic activity. *J. Biol. Chem.* 270:16871-16878.