Hepatocyte Growth Factor/Scatter Factor Binds to Small Heparin-derived Oligosaccharides and Stimulates the Proliferation of Human HaCaT Keratinocytes*

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Hepatocyte growth factor/scatter factor (HGF/SF) acts via a dual receptor system consisting of the MET tyrosine kinase receptor and heparan sulfate or dermatan sulfate proteoglycans. In optical biosensor binding assays, competition by oligosaccharides for binding of HGF/SF to immobilized heparin showed that disaccharides failed to compete, whereas tetrasaccharides inhibited HGF/SF binding (IC50 8 µg/ml). The inhibitory potency of the oligosaccharides increased as their length increased by successive disaccharide units, to reach a maximum (IC50 1 µg/ml) at degree of polymerization (dp) 10. In binding assays, HGF/SF was found to bind directly to oligosaccharides as small as dp 4, and the binding parameters were similar for oligosaccharides of dp 4–14 (Kd 2.2–45.3 × 108 M–1 s–1, k2 0.033–0.039 s–1, and Kf 9–16 nm). In human keratinocytes, HGF/SF stimulated DNA synthesis, and this was dependent on a sustained phosphorylation of p42/44 MAPK. In chlorate-treated and hence sulfated glycosaminoglycan-deficient HaCaT cells, the stimulation of DNA synthesis by HGF/SF was almost abolished. Heparin-derived oligosaccharides from dp 2 to dp 24 were added together with HGF/SF to chlorate-treated cells to determine the minimum size of oligosaccharides able to restore HGF/SF activity. At restricted concentrations of oligosaccharides (4 ng/ml), HGF/SF required decasaccharides, whereas at higher concentrations (100 ng/ml) even tetrasaccharides were able to partly restore DNA synthesis. The results suggest that HGF/SF binds to a tetrasaccharide and that although this is sufficient to enable the stimulation of DNA synthesis, longer oligosaccharides are more efficient, perhaps by virtue of their ability to bind more easily other molecules.

HGF/SF is a well described heparan sulfate (HS) and dermatan sulfate (DS) binding growth factor with mitogenic, morphogenic, and motogenic activities toward many normal and neoplastic epithelial cells (1, 2), as well as at least some stromal cells (3). In vivo, HGF/SF mediates epithelial-mesenchymal interactions, which are crucial for embryonic development, as well as tissue regeneration processes (4). By virtue of its mitogenic and angiogenic activities, HGF/SF is involved in tumorogenesis and metastasis (5–8). The diverse biological effects of HGF/SF are transduced by activation of its transmembrane receptor MET, encoded by the c-met protooncogene (1, 9). Binding of HGF/SF, which is thought to induce MET dimerization and autophosphorylation, activates multiple signaling cascades (1). Two naturally occurring HGF/SF isoforms, which consist of the N-terminal domain and either the first kringel repeat (NK1) or the first two kringle repeats (NK2), bind MET and HS/heparin and function as agonists or antagonists of the full-length HGF/SF (10–14).

The interactions of HGF/SF with HS and DS are of high affinity, with Kd values ranging from 0.2 to 20 nM (15, 16). By combining scission of HS and DS with sequence-specific enzymes and affinity chromatography on HGF/SF, a minimal binding sequence in HS was approximated to [IdoA-GlcNS(6-O-OSO3)3]3 (17) and in DS to [IdoA-GalNac4-OSO3]3 (15). Although the absolute number and positioning of critical residues is unknown, these analyses demonstrate the critical importance of the idurionate residues themselves, because chondroitin sulfate, similarly sulfated to DS but lacking iduronate, fails to bind HGF/SF. Moreover, it is clear that both N-sulfation of hexosamine and 2-O-sulfation of idurionate play no role in HGF/SF binding (15). Heparin was shown to be required for NK1 to increase the tyrosine phosphorylation of MET and downstream signaling in mutant Chinese hamster ovary cells devoid of HS and DS (11). However, in hematopoietic cells lacking HS, heparin potentiated the activity of HGF/SF and NK1 but was not absolutely required for HGF/SF (12, 14). In contrast, the activity of HGF/SF is dependent on HS or on DS in mutant Chinese hamster ovary cells deficient in these GAGs (18). Moreover, in cell systems rendered deficient in sulfated GAG by treatment with chlorate, the mitogenic and motogenic responses to HGF/SF are strongly dependent on the presence of GAG (19, 20). Heparin-binding sites have been identified in the N-terminal domain of HGF/SF, as well as in kringel domains 1 and 2 (11, 21–25).

Therefore, the balance of evidence favors a model in which the diverse biological activities of HGF/SF are dependent upon the presence of GAG to which it can bind. Oligomerization of

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‡ The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; DS, dermatan sulfate; dp, degree of polymerization; GAG, glycosaminoglycan; HS, heparan sulfate; MAPK, mitogen-activated protein kinases; PBS, phosphate-buffered saline; RSK, ribosomal subunit S6 kinase.

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An inhibitor of mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase, PD098059 (2′-2′-amino-3′-methoxyphenyl)oxanaphthalen-4-one (34–36) diluted in Me2SO was added directly to the medium at a concentration of 50 μM and 15 min before the addition of the growth factors. At the times indicated in the figure, cells were washed with ice-cold PBS, blocked for 30 min in 300 μl of lysis buffer (50 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.06% bromphenol blue (w/v), 2% (v/v) β-mercaptoethanol, and protease inhibitor mixture), scraped with a rubber policeman, and collected in 1.5-ml Eppendorf tubes. All steps were performed at 4 °C. Identical amounts of protein were separated by SDS-PAGE. After transfer to nitrocellulose, the membranes were incubated in a blotting solution (Tris-buffered saline containing 5% (v/v) nonfat dry milk; 0.05% (v/v) Tween 20). Incubation with the primary antibody diluted at 1:500 was carried out overnight at 4 °C in the blotting solution. After 5 washes in Tris-buffered saline containing 0.05% (v/v) Tween 20, the nitrocellulose membrane was incubated with secondary peroxidase-conjugated antibodies to IgG antibodies, diluted 1:1000 in the blotting solution. Following several washes with Tris-buffered saline containing 0.05% (v/v) Tween 20, immunoreactive proteins were revealed with the SuperSignal chemiluminescent detection system (Pierce and Warriner, Chester, UK) on Hyperfilm (Amersham Biosciences).

**Immobilization of Oligosaccharides**—Porcine intestinal mucosal heparin was biotinylated on amine groups with N-hydroxysuccinimide (LC)-biotin from Pierce and Warriner (Chester, UK). The pan extracellular signal-regulated kinase antibody, the antibodies against the phosphorylated forms of p90 RSK, and the antibodies against the dually phosphorylated Thr(183/202) Tyr(185/204) forms of p42/44 MAPK were purchased from New England Biolabs (Hitchin, UK). Secondary peroxidase-labeled anti-IgG antibodies were purchased from Pierce (Rockford, IL). Secondary peroxidase-labeled anti-IgG antibodies were purchased from SuperSignal (Pierce Biotechnology, Inc., Rockford, IL). Secondary peroxidase-labeled-anti-IgG antibodies were from Amersham Biosciences.

**Measurement of DNA Synthesis**—The immortalized human HaCaT keratinocytes (generously provided by Dr. N. Fusenig, Germany) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and the antibiotics penicillin (1000 units/ml) and streptomycin (1 mg/ml) (Invitrogen) (27). Cells were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37 °C

**Experimental Procedures**

**Materials**—Human recombinant HGF/SF was obtained from R & D Systems (Abington, UK). Heparin-derived oligosaccharides (dp 4–26), prepared from partial heparinase I digests of pig mucosal heparin, were obtained from Iduron (Manchester, UK); the main disaccharide unit in these saccharides (>75%) being IdoA2S-GlcNS6S. Porcine intestinal mucosal heparin, trisulfated heparin disaccharide, and streptavidin were from Sigma. All reagents for electrophoresis were purchased from Bio-Rad. PD098059 and biotin-XX-hydrizide were from Calbiochem, and N-hydroxysuccinimide (LC)-biotin was from Pierce and Warriner (Chester, UK). The pan extracellular signal-regulated kinase antibodies, which recognize p42/44 MAPK regardless of their state of phosphorylation, the antibodies against the phosphorylated forms of p90 RSK, and the antibodies against the dually phosphorylated Thr(183/202) Tyr(185/204) forms of p42/44 MAPK were purchased from New England Biolabs (Hitchin, UK).

**RESULTS**

**Phosphorylated Forms of p42/44MAPK and p90RSK by Western Blotting**—Cells were seeded at equal densities in 10-cm diameter cultured dishes and then treated identically as for the DNA synthesis assay up to the addition of growth factors. In some experiments
FIG. 1. Competition of HGF/SF binding to immobilized heparin by oligosaccharides of different lengths. The extent of binding of HGF/SF to heparin immobilized on an aminosilane surface was measured in the presence of increasing concentrations of oligosaccharides of different lengths (“Experimental Procedures”). Maximal binding of HGF/SF in the absence of competing oligosaccharides was 80%. The amount of HGF/SF bound to the immobilized heparin at each concentration of oligosaccharide was calculated as a percentage of this maximal value. Errors for individual datum points were less than 1% of the mean and are omitted for clarity. Similar results were obtained in three separate experiments.

210 s. The cuvette was then washed three times with 50 μl of PBST, and the dissociation of bound ligate into the bulk PBST was followed over time. The surface was regenerated by washing twice with 50 μl of 2 M NaCl, 10 mM phosphate, pH 7.2. Binding parameters were calculated from the association and dissociation phases of the binding reactions using the non-linear curve-fitting FastFit software (Affinity Sensors). A single binding assay yielded four binding parameters as follows: the initial rate of association, the on-rate constant (k_{on}), and the extent of binding, all calculated from the association phase, and the off-rate constant (k_{off}) equivalent to the dissociation rate constant, k_{diss}, calculated from the dissociation phase. In these kinetic experiments, k_{on} was only determined at low concentrations of HGF/SF, whereas k_{off} was measured both at these lower concentrations of HGF/SF and, in separate experiments, using higher concentrations of HGF/SF and competing heparin (100 μg/ml) in the dissociation buffer to avoid any rebinding artifacts (38, 39). The equilibrium dissociation constant (K_{D}) was calculated from the ratio of the association and dissociation rate constants. A single site binding model fitted the data at least as well as a two-site binding model in both the competitive binding assays and the kinetic experiments. Therefore the binding reaction between HGF/SF and the oligosaccharides was deemed to be monophasic, and a single site model was used to calculate all binding parameters.

RESULTS

Competition by Heparin-derived Oligosaccharides for HGF/SF Binding to Heparin—The ability of soluble oligosaccharides to compete with immobilized heparin for HGF/SF binding was determined in an optical biosensor. Disaccharides (dp 2) in solution failed to inhibit the binding of HGF/SF to the heparin immobilized on the biosensor cuvette (Fig. 1), even at 333 μg/ml (result not shown). Surprisingly, tetrasaccharides competed efficiently for HGF/SF binding to the immobilized heparin and inhibited 50% of the binding of HGF/SF (IC_{50} at 8 μg/ml (Fig. 1). The competition by oligosaccharides for HGF/SF binding to heparin exhibited a size-dependent gradation. The shortest competing oligosaccharides (dp 4) were the least potent (IC_{50} 8 μg/ml), and as the oligosaccharides progressively increased in length by a disaccharide unit to dp 10, there was an increase in the potency of their inhibition of HGF/SF binding to heparin (dp 10 with an IC_{50} of 1 μg/ml). Heparin was slightly more potent (IC_{50} 0.7 ng/ml) than the decasaccharide.

Kinetics of HGF/SF Binding to Heparin-derived Oligosaccharides—The kinetics of the interaction of HGF/SF with re-
immortalized keratinocytes that maintain the ability to differentiate in culture (27, 41–43). HGF/SF strongly stimulated DNA synthesis in these cells (Fig. 3A), which possess the MET tyrosine kinase receptor and heparan sulfate (data not shown). Addition of PD098059, a well-established inhibitor of mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase-1, 15 min before the addition of HGF/SF reduced DNA synthesis to the level seen in untreated cells. Moreover, after addition of PD098059, a near complete inhibition of DNA synthesis (result not shown). Therefore, in HaCaT keratinocytes (Fig. 4), whereas heparin alone had no effect on the growth-stimulatory effects of HGF/SF in chlorate-treated keratinocytes (Fig. 4), these cells provide a sensitive assay for studying the dependence of the stimulation of cell proliferation by HGF/SF on these cells. Chlorate is a potent inhibitor of sulfation, widely used to address sulfated GAG function (19, 20, 28–32, 44). When HaCaT keratinocytes are grown in the presence of 15 mM sodium chloride, DNA synthesis induced by HGF/SF is strongly reduced (Fig. 4). Chlorate itself did not affect the intrinsic ability of the cells to trigger a growth-stimulatory response, because dialyzed serum stimulated DNA synthesis in chlorate-treated HaCaT cells (Fig. 4). Moreover, addition of 7.5 mM Na₂SO₄, which will relieve the inhibition of 3-phosphoadenosine 5-phosphosulfate synthesis by 15 mM chloride (30, 31) and hence enable the synthesis of sulfated glycosaminoglycan chains by the cells, restored the growth-stimulatory effect of HGF/SF (Fig. 4). The addition of soluble heparin (10 ng/ml) simultaneously with HGF/SF also restored the growth-stimulatory effects of HGF/SF in chlorate-treated keratinocytes (Fig. 4), whereas heparin alone had no effect on DNA synthesis (result not shown). Therefore, in HaCaT keratinocytes, HGF/SF requires the presence of heparan sulfate receptor to fully trigger a proliferative response.

Effect of Heparin-derived Oligosaccharides on HGF/SF Stimulation of DNA Synthesis—The stimulation of proliferation of HaCaT by HGF/SF is dependent on sulfated GAGs, and the growth stimulatory activity of HGF/SF in chlorate-treated HaCaT cells is restored by the addition of soluble heparin. Thus, these cells provide a sensitive assay for studying the dependence of the stimulation of cell proliferation by HGF/SF on GAGs. Heparin-derived oligosaccharides from dp 2 to 24 were used with chlorate-treated cells to determine the minimum size of oligosaccharides able to restore HGF/SF-stimulated DNA synthesis (Fig. 5). The trisulfated disaccharide was without effect at all concentrations tested (Fig. 5). In contrast, tetrasaccharides were able to restore partly the growth stimulatory activity of HGF/SF, and their ED₅₀ was ~100 ng/ml (Fig. 5).
DISCUSSION

HGF/SF binds to two distinct types of receptors, a transmembrane tyrosine kinase, MET, which corresponds to the product of the c-met protooncogene, and proteoglycans bearing chains of the glycosaminoglycans HS or DS. HGF/SF, like many GAG-binding growth factors, must interact with both types of receptor to promote a cellular response (2, 11, 18–20, 45). However, the mechanism by which the HS receptors or the more recently described DS receptors contribute to the delivery of growth-stimulatory signals by HGF/SF is unclear. To determine some of the limiting structural features in HS/heparin that enable HGF/SF to stimulate cell proliferation, we used optical biosensor-based binding assays to analyze the interaction between HGF/SF and either soluble or reducing end immobilized oligosaccharides in competition and direct binding studies, respectively. The competition assays show that tetrasaccharides contain all the necessary information required for HGF/SF binding, because they clearly inhibit the binding of HGF/SF to heparin. However, as the length of the oligosaccharide increases by successive disaccharide units to dp 10, the efficiency of inhibition of HGF/SF binding to heparin increases. The measurement of the kinetics of HGF/SF binding to immobilized oligosaccharides provides a quantitative analysis of these interactions. Surprisingly, in contrast to the competition experiment (Fig. 1), the results (Table I) show that there is no difference in the association rate constant, \( k_a \), the dissociation rate constant, \( k_d \), and the equilibrium dissociation constant, \( K_d \), of HGF/SF for the oligosaccharides of dp 4–14. It seems unlikely that the longer oligosaccharides, which are competing more effectively for HGF/SF, are able to bind more than one molecule of HGF/SF, because the steric hindrance attributable to multivalent binding would have resulted in biphasic binding kinetics at higher concentrations of HGF/SF (38). An explanation for the higher efficiency of HGF/SF binding by the longer oligosaccharides in the competition assay is that they may present, in solution, more opportunities for a collision with HGF/SF to be productive and result in a binding event. However, when the same oligosaccharides are immobilized on the planar surface of the biosensor, they all have the same orientation, and so this effect of length would no longer be apparent. Moreover, because the HGF/SF binding kinetics do not vary with the length of the oligosaccharides, this suggests that the binding site of HGF/SF in heparin is equivalent to the shortest oligosaccharide, dp 4. However, the non-reducing terminal hexuronic acid of the oligosaccharides will be 4,5-unsaturated because of the action of heparinase I, whereas the biotinylation reaction will cause the reducing terminal GlcN unit to lose its ring structure. Therefore, only part of the oligosaccharides, including the minimal tetrasaccharide, used in this work represent the native structure.

To gain insights into the relationship of oligosaccharide length and biological potency, we then developed a model based on human HaCaT keratinocytes. The proliferation of these cells is stimulated by HGF/SF (Fig. 3). In the presence of chlorate, which inhibits sulfation on proteins and on carbohydrate residues in intact cells without inhibiting cell growth or protein synthesis (29–32), the growth-stimulatory response of HaCaT cells to HGF/SF is strongly reduced. Moreover, the addition of soluble heparin restores the growth stimulatory activity of HGF/SF. Thus, as observed in other cell types (18–20, 40), the cellular response to HGF/SF in HaCaT keratinocytes depends on the presence of sulfated glycosaminoglycans such as HS and DS. Interestingly, in some other epithelial cell systems, the exogenously added HS must be anchored to the substratum to restore the cellular response to HGF/SF (19, 20). The observation that soluble heparin is functional in the present assay makes HaCaT cells a more tractable model for studying HGF/SF-dependent structure-function relationships in GAGs.

When soluble oligosaccharides of different lengths were tested for their ability to restore the growth stimulatory activity of HGF/SF in chlorate-treated HaCaT cells, we observed that tetrasaccharides were the shortest oligosaccharides active in this assay. Previous work has suggested that longer oligosaccharides are required. For example, it has been reported that the stimulation of migration of mutant Chinese hamster ovary cells devoid of HS and DS requires oligosaccharides of at least dp 6 (18). In addition, semisynthetic sulfated oligosaccharides of dp 6, but not dp 4, potentiated the activity of HGF/SF in cells containing GAGs (40). The \( K_{50} \) values of oligosaccharides of dp 4, 6, and 8 were similar in our study, but between dp 8 and 14 there was a clear increase in potency associated with the increasing length of the oligosaccharides. Therefore, at the sub-optimal concentrations of oligosaccharides used in this as-

**FIG. 4.** Effect of chlorate treatment of HaCaT cells on HGF/SF-stimulated cell proliferation. DNA synthesis assays were carried out as described under “Experimental Procedures.” HaCaT cells were cultured for 24 h in sulfate-free medium containing 15 mM sodium chlorate and were then incubated with HGF/SF (30 ng/ml), serum 10% (v/v), heparin (10 ng/ml), or \( \text{Na}_2\text{SO}_4 \) (7.5 mM) as indicated. Results are the mean ± S.D. of three experiments.

**FIG. 5.** Stimulation of DNA synthesis in chlorate-treated HaCaT cells by HGF/SF in the presence of increasing concentrations of heparin-derived oligosaccharides. DNA synthesis assays were carried out as described under “Experimental Procedures.” HGF/SF (30 ng/ml) was added to serum-starved chlorate-treated HaCaT keratinocytes along with increasing concentrations of heparin-derived oligosaccharides of different lengths. Results are the mean of triplicate wells of one of four experiments. The S.D. was less than 10% of the mean and is omitted for clarity.

Oligosaccharides of dp 6 and 8 had the same potency in this assay as the tetrasaccharides. However, increasing the length of the oligosaccharide by successive disaccharide units to dp 14 resulted in a substantial increase in potency, with an \( \text{ED}_{50} \) of 20 ng/ml for dp 10, 4 ng/ml for dp 12, and around 1 ng/ml for dp ≥ 14, which was the maximal potency observed in this assay (Fig. 5).
say, below dp 14 the length of the oligosaccharide becomes limiting with respect to the ability of HGFSF to stimulate cell proliferation.

The structure of HGFSF-binding sites in HS and DS have been partially elucidated. In HS, HGFSF interacts within the S domains (17), and binding appeared to require at least a hexasaccharide by affinity chromatography (17, 46). A combination of iduronates and 6-O-sulfation appears to be critical, although not N-sulfation (17) or 2-O-sulfation (47). The minimal binding sequence in HS was approximated to [IdoA-GlcNS(6-OSO3)]3, although the absolute number and positioning of critical residues are unknown. The analysis of the binding site of HGFSF in DS has confirmed that N-sulfation of hexosamine and 2-O-sulfation of idurionate play no role in HGFSF binding (15). However, the latter study underlines the critical importance of the idurionate residues themselves, because chondroitin sulfate, similarly sulfated to DS but lacking idurionate, fails to bind HGFSF. Thus the minimum binding sequence for HGFSF in DS is likely to be [IdoA-GalNAc(4-OSO3)]3. The question arises as to why the present quantitative measurements show that the HGFSF-binding site in heparin is a tetrasaccharide. The 6-O-sulfate groups in HS are thought to be essential for HGFSF binding (17). Sequencing of heparinase III-released S domains from 3T3 cell-derived HS has revealed that they are sparingly 6-O-sulfated, and a significant number are completely deficient in 6-O-sulfate groups. For example, only 12% of sequenced hexasaccharides and 29% of sequenced octasaccharides contained a 6-O-sulfate group (48). Therefore, it is likely that oligosaccharides derived from HS that bind HGFSF will be longer than dp 4 to contain sufficient 6-O-sulfate groups. Moreover, a recent study (49) on a novel HGFSF-binding PG isolated from endothelial cells, endocan, shows that it carries DS chains with a small number (1-2) of iduronates per chain. Although the sequential position of these iduronates is unknown, this observation supports the contention that HGFSF may only require a very short sequence of iduronates possibly in oligosaccharides as small as dp 4.

A recent report (22) established that heparin binding does not induce a conformational change in NK1, but, as suggested by previous work (12, 26), heparin promotes the formation of the NK1 dimer. However, such studies employ a subdomain of HGFSF in isolation. The present results show that heparin-derived oligosaccharides as short as dp 4 are able to replace cellular HS in terms of enabling HGFSF to stimulate keratinocyte proliferation. Because the binding parameters of HGFSF to the oligosaccharides of different length are the same, this suggests that a tetrasaccharide is the unit that is recognized by HGFSF. Given the large size of HGFSF (84 kDa, ~7 nm diameter for a globular protein) compared with other HS-binding growth factors, e.g., fibroblast growth factor-2 (18 kDa, ~4.5 nm diameter), it seems unlikely that a tetrasaccharide (length ~1.6 nm) can support dimerization of HGFSF in cis mode. However, the observation that increasing the length of oligosaccharides by successive disaccharide units from dp 8 to 14 (length ~5.6 nm) increases their biological potency could be interpreted as representing the more efficient dimerization of HGFSF by the oligosaccharide. There is also the possibility of HGFSF dimerization in trans mode. However, a recent study using biologically active 1:1 covalent monomeric complexes of HS with DS oligosaccharides of dp 8–12 indicates that dimerization per se is unlikely to play a significant role in the mechanism whereby these GAGs enable the cellular response to HGFSF (18). The binding of HGFSF to the longer oligosaccharides (dp 10 and 14) was monophasic; if such oligosaccharides could support HGFSF dimerization, biphatic kinetics would have been expected at the higher concentrations of HGFSF due to steric hindrance (38). Taken these results suggest that, whereas small oligosaccharides can display activity, the maximal attainable biological activity of oligosaccharides may require an interaction with HGFSF and another protein, which is facilitated when dp >8. One suggestion for the identity of the other protein is MET itself, which has been shown to bind to heparin (14, 22), although another report queries whether the interaction of the native MET extracellular domain is of significant affinity by itself (18). However, given the high affinity of the interaction between HGFSF and GAG, putative additional GAG-MET interactions would not need to be of great affinity. The structural features in HS required for this interaction and whether these requirements are compatible with those necessary for HGFSF binding are currently unknown.

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