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Insights into the Structure/Function of Hepatocyte Growth Factor/Scatter Factor from Studies with Individual Domains

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Hepatocyte growth factor/scatter factor (HGF/SF), the ligand for the receptor tyrosine kinase encoded by the c-Met proto-oncogene, is a multidomain protein structurally related to the pro-enzyme plasminogen and with major roles in development, tissue regeneration and cancer.[1] We have expressed the N-terminal (N) domain, the four kringle domains (K1 to K4) and the serine proteinase homology domain (SP) of HGF/SF individually in yeast or mammalian cells and studied their ability to: (i) bind the Met receptor as well as heparan sulphate and dermatan sulphate co-receptors, (ii) activate Met in target cells and, (iii) map their binding sites onto the β-propeller domain of Met. The N, K1 and SP domains bound Met directly with comparable affinities (K_d = 2.4, 3.3 and 1.4 μM). The same domains also bound heparin with decreasing affinities (*N* > K1 >> SP) but only the N domain bound dermatan sulphate. Three kringle domains (K1, K2 and K4) displayed agonistic activity on target cells. In contrast, the N and SP domains, although capable of Met binding, displayed no or little activity. Further, cross-linking experiments demonstrated that both the N domain and kringles 1-2 bind the β-chain moiety (amino acid residues 308–514) of the Met β-propeller. In summary, the K1, K2 and K4 domains of HGF/SF are sufficient for Met activation, whereas the N and SP domains are not, although the latter domains contribute additional binding sites necessary for receptor activation by full length HGF/SF. The results provide new insights into the structure/function of HGF/SF and a basis for engineering the N and K1 domains as receptor antagonists for cancer therapy.

Keywords: HGF/SF; c-Met; receptor tyrosine kinase; glycosaminoglycans

Introduction

Hepatocyte growth factor/scatter factor (HGF/SF) and its homologue hepatocyte growth factor-like/macrophage stimulating protein factor (HGFl/MSP) are two polypeptide growth factors with major roles in development and tissue regeneration in vertebrate organisms. Genetic experiments in the mouse have demonstrated that HGF/SF and its receptor Met, the tyrosine kinase encoded by the c-Met proto-oncogene, provide signals essential for the development of the placenta, liver, tongue, diaphragm, limb muscles and certain groups of neurons.[3–5] Further, experiments with conditional *Met* alleles have established that HGF/SF and Met are required in post-natal life for liver regeneration[6,7] and the survival and activity of the endocrine pancreas.[8] HGF/SF and Met have also been widely implicated in human cancer due to activating mutations[9] or overexpression of the receptor.[10,11] The essential roles of HGF/SF and Met in vertebrate biology and their major roles in human disease have

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Abbreviations used: aa, amino acid; AMAC, 2-aminoacridone; BS^3, bis[sulfosuccinimidyl]suberate; DS, dermatan sulphate; ERK, extracellular signal-regulated protein kinase; GAG, glycosaminoglycan(s); HGF/SF, hepatocyte growth factor/scatter factor; HGFI/MSP, hepatocyte growth factor-like/macrophage stimulating protein; HS, heparan sulphate.

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generated considerable interest in the structural basis of Met signalling and in utilising this knowledge for the development of Met-based therapeutics.

Unlike other known polypeptide growth factors, which typically are small and single-domain proteins, HGF/SF is a large, multi-domain protein of 728 amino acid residues related to the blood proteinase precursor plasminogen. Like plasminogen, HGF/SF is produced as an inactive protein (pro- or single-chain HGF/SF) which is subsequently cleaved at a trypsin-like site by plasminogen activators[12] or a serine proteinase related to coagulation factor XII.[13] Processing of single-chain HGF/SF yields a two-chain, mature form consisting of disulphide-linked α and β-chains of ~69 kDa and ~34 kDa, respectively.[14,15] The α-chain consists of an N-terminal domain related to the so-called plasminogen activation peptide and four copies of the kringle domain (K1 to K4). The β-chain consists of a single domain that retains the fold of the catalytically active serine proteinases (SP) but has no enzymatic activity due to mutations in catalytic residues and in the S1 specificity pocket.[2]

The modular architecture of HGF/SF is ideally suited for protein engineering experiments and enabled early insights into structure/function. HGF/SF mutants in which the N, K1 or SP domains had been ablated are biologically inactive, whereas mutants lacking the K2, K3 or K4 domains retain varying degrees of biological activity.[16,17] The interpretation of these results, however, may be confounded by secondary effects of the mutation(s), such as effects on the distance and/or orientation of residual domains.

Separate progress in structure/function analysis of HGF/SF has been achieved through several crystal structures of NK1 (a fragment containing the N-terminal and K1 domains),[18–20] the SP domain[21] and the SP domain in complex with a fragment of the Met receptor.[22] The conclusions of these studies are that two-chain HGF/SF binds the Met receptor using the K1 and SP domains and that both these domains are necessary for receptor activation. Yet, the structural basis of Met activation remains incompletely understood and either the SP or N/K1 domains have been implicated in receptor dimerisation on the basis of crystal contacts[22] or low-resolution structures of HGF/SF-Met complexes obtained by cryo-EM or small angle X-ray scattering.[23]

In addition to Met, HGF/SF also interacts with both heparan sulphate (HS)[24] and dermatan sulphate (DS).[25,26] These glycosaminoglycans (GAGs) function as co-receptors[27] potentiating HGF/SF-mediated activation of Met on target cells. The shortest oligosaccharides that bind and possess activity are tetra- and hexa-saccharides of HS and DS, respectively.[28,29] The very similar GAG-binding properties of HGF/SF and NK1 suggest that the major GAG-binding site is restricted to within the NK1 region of the protein,[29] consistent with the predominant interactions with the N-domain seen in the crystal structure of complexes between NK1 and a heparin dodecasaccharide.[30]

In order to gain new insights into the structure/function of HGF/SF we have expressed the six domains of HGF/SF individually and studied their ability, and that of selected domain combinations, to: (i) bind the Met receptor as well as HS/DS co-receptors, (ii) activate Met in target cells and, (iii) form ligand-receptor complexes with soluble Met fragments in the absence or presence of co-receptors. The study defines the relative role of single HGF/SF domains in receptor binding and/or activation and in binding HS/DS co-receptors. It also offers a rationale for engineering the N or K1 domains as receptor antagonists.

Results

Binding of HGF/SF domains to Met and GAG co-receptors

The N, K1, K2, K3, K4 domains and the NK1 and NK2 fragments of HGF/SF were expressed in the yeast *Pichia pastoris*; the SP domain and the NK3, NK4 and K4-SP fragments were produced either in Chinese hamster ovary (CHO) cells or in the mouse myeloma line NS0. All constructs were purified first by metal-chelate chromatography *via* a hexa-histidine tag fused at the C terminus, followed by gel filtration chromatography (Figure 1(a)). Correct folding of HGF/SF domains was confirmed by velocity sedimentation experiments and representative results are shown in Figure 1(b)–(d) for the N, K1 and SP domains, respectively. Plots of $g(s^*)$ against $s^*_{20,w}$ are shown on the left and plots of the residuals, from fitting models to the data, against $s^*_{20,w}$ on the right. At the micromolar concentrations used, the N domain existed in a monomer-dimer equilibrium yielding a mean $M = 14.1$ kDa for the monomeric species, against an M of 12.0 kDa calculated from sequence (data not shown). The K1, K2, K3 and SP domains were monomeric and yielded $M = 10.3(\pm 0.7)$, $10.2(\pm 0.3)$, $9.1(\pm 0.2)$ and $29.7(\pm 0.5)$ kDa, respectively against values of 11.1, 11.5, 11.4 and 26.8 kDa respectively calculated from sequence. Velocity sedimentation analysis of K4 produced unreliable results due to hyperglycosylation of N_{402}, as observed with other proteins carrying N-glycosylation sequences and expressed in *P. pastoris*.[31] The domain was used nevertheless in subsequent studies because deglycosylation with Endo H yielded a protein of the correct size (Figure 1(a)) and because all other kringle domains expressed in *P. pastoris*, and lacking N-glycosylation sequences, were correctly folded. Further characterisation of N and K1–K4 domains of HGF/SF by mass spectrometry (MALDI-TOF) confirmed their expected molecular masses and the absence of carbohydrate side-chains in the N, K1, K2 and K3 domains (data not shown).

Direct binding of individual HGF/SF domains to Met was studied by surface plasmon resonance using a soluble fragment of the Met ectodomain (Met567, amino acid residues 25–567) on the chip.[22]

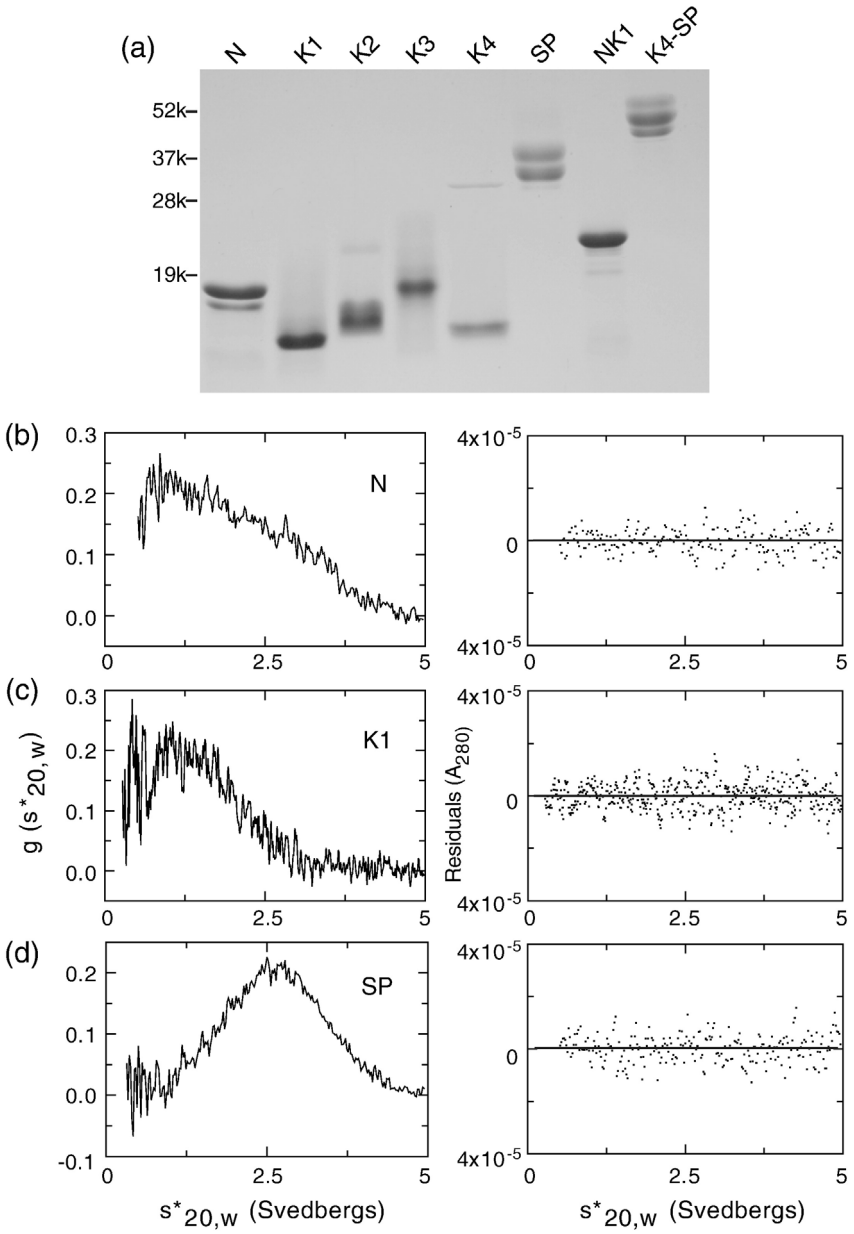

Figure 1. (a) Non-reducing SDS-PAGE of HGF/SF domains and multidomain fragments. Prior to running of the gel, the K4 domain was incubated with EndoH (the faint band of ~32 kDa) at 30 °C for 5 h. (b)–(d) Velocity sedimentation analysis of the N, K1 and SP domains. The left panels show plots of $g(s^*_{20,w})$ against $s^*_{20,w}$, the right panels show plots of the residuals, from fitting models to the data, against s*20,w. A two-component model was used for the N domain, and one-component models were used for the K1 and SP domains.

This Met fragment contains the β-propeller domain (amino acid residues 25–514) shown to be necessary and sufficient to bind HGF/SF.[32] NK1 bound Met567 with a $K_d = 0.15$ μM (Figure 2(a)) and the K1 and SP domains bound Met567 with $K_d = 3.3$ μM and 1.4 μM, respectively (Figure 2(c) and (g)). Met binding by the K2, K3 and K4 domains was weaker (Figure 2(d)–(f)) and did not enable accurate calculations of kinetic and equilibrium constants. In contrast, the N domain bound Met567 with a $K_d = 2.4$ μM, a value comparable to those of the K1 and SP domains (Figure 2(b)). Thus the higher affinity for Met of the α-chain of HGF/SF compared to the β-chain is not due to tighter binding by the K1 domain but by the fact that two domains (N and K1) contribute to binding with similar affinities. Experiments in which equimolar concentrations of N + SP, K1 + SP or N + K1 domains were exposed to the Met chip demonstrated simultaneous binding of the N + SP or K1 + SP domains to Met (Figure 2(i)). Simultaneous binding of the N + K1 domains could not be demonstrated (Figure 2(i)), probably due to the fact that the orientation of the two domains may have to be restrained, as in NK1,[18–20] in order for them to bind simultaneously.

HS is essential for biological activity of the truncated splice variants NK1 and NK2[33] and binding to HS/DS potentiates the activity of full length HGF/SF on certain target cells.[27,33–35] The binding of individual HGF/SF domains to DS and heparin, a structural analogue of HS, was studied by gel shift and chromatographic experiments (Figures 3 and 4). The unusual ability of HGF/SF[25,26] or NK1[29] to bind DS dp12 with high affinity was mapped to the N domain in gel shift experiments (Figure 3(a)). Thus HGF/SF contains a single binding site for DS, located in the N domain. In contrast, earlier domain-deletion experiments had indicated the presence of two binding sites for heparin in the N

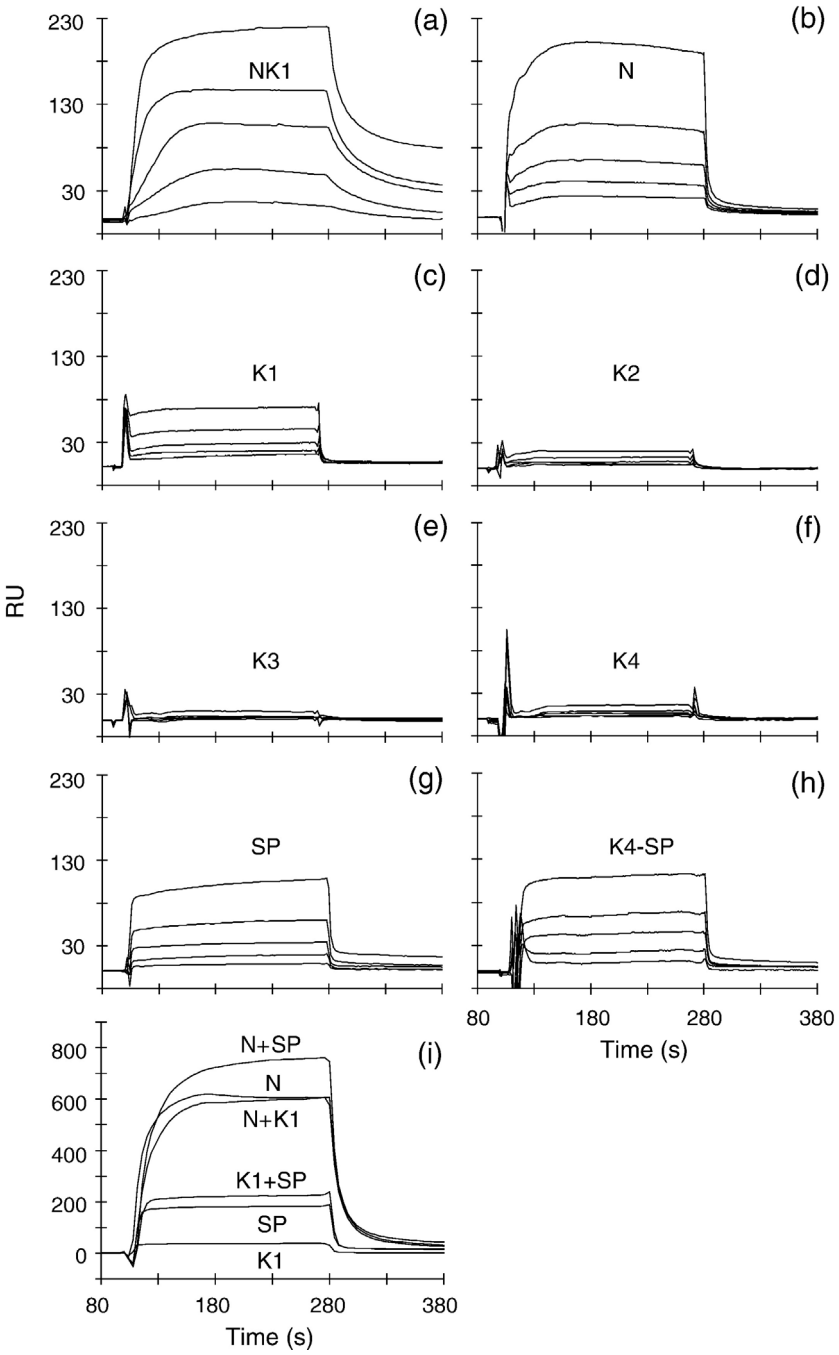

Figure 2. Binding of HGF/SF domains and multidomain fragments to Met567 using surface plasmon resonance. (a)–(h) Two-fold dilutions of each protein were used from a concentration of 200 nM for NK1 (a), 1 μM for the N domain (b), 4 μM for K1 to K4 ((c)–(f)), and 500 nM for the SP domain (g) and the K4-SP fragment (h). (i) Binding of single domains and domain combinations to Met567 using surface plasmon resonance. Concentrations used are 4 μM for the N and K1 domains and 1 μM for the SP domain; 60 μl of analyte was injected followed by a 300 s dissociation with buffer alone.

and K2 domains of HGF/SF,[36] a result confirmed by site-directed mutagenesis experiments.[37] Gel shift experiments with heparin dp12 showed that the N, K1 and SP domains, but not K2, K3 or K4, bound heparin (Figure 3(b)). Further titrations with shorter heparin dp4 fragments at different protein/heparin molar ratios are shown in Figure 4(b)–(d) for the N, K1 and SP domains and compared with NK1 (Figure 4(a)). These experiments, as well as salt-elution from a heparin-Sepharose column (Figure 4(e)), established the relative binding affinities for heparin of the three domains as: N > K1 >> SP.

The ability of heparin dp12 to induce domain oligomerisation was studied next. Cross-linking experiments with BS^3 had shown that heparin induces dimerisation of NK1[30,33,38] and the N domain, but not K1,[38] hence a similar approach was applied here to all HGF/SF domains. The SP and, to a lesser extent, the K2 and K3 domains showed a clear tendency to dimerise in the absence of heparin and addition of heparin had no further effect on oligomerisation of these domains (Figure 5(a)). Heparin caused dimerisation/oligomerisation of the N domain but not of the heparin-binding K1 domain (Figure 5(a)), a result consistent with an earlier study[38] and confirmed by velocity sedimentation analysis of K1-heparin dp12 complexes (data not shown). The ability of heparin dp12 to modulate the binding of the N and K1 domains to the Met receptor was studied next by gel filtration. In the case of the N

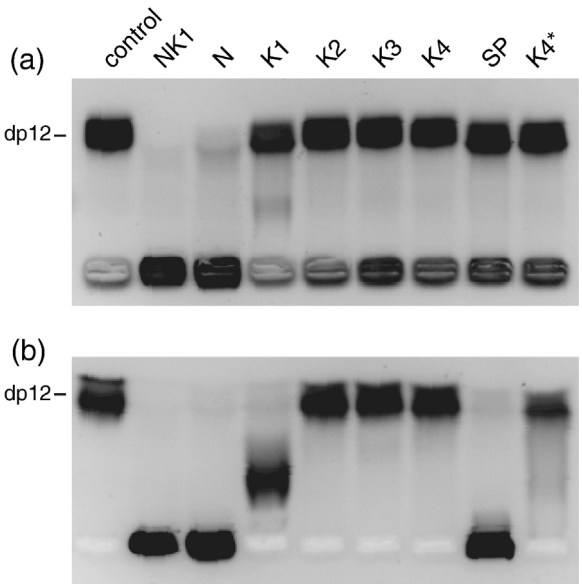

Figure 3. Binding of HGF/SF domains to DS (a) or heparin (b) by gel mobility shift assay. Protein domains were at 100 μM and were incubated with 28 μM AMAC-dp12 fragments of DS or heparin prior to electrophoresis.

domain, heparin induced the formation of: (i) large ligand-receptor complexes (M_r > 1,300,000) eluting in the void volume, (ii) small amounts of an N domain-Met567 complex co-eluting with the main Met567 peak (as shown by slot blot analysis of the relevant fractions) and, (iii) a small peak corresponding to an N domain-heparin dp12 complex (Figure 5(b)). In contrast, in the case of the K1 domain addition of heparin dp12 caused the formation of a complex with a M_r of ~350,000 (Figure 5(c)). Heparin dp12 had no effect on the elution profile of Met567 in the absence of HGF/SF domains (Figure 5(d)).

Biological activity of HGF/SF domains and selected domain combinations

HGF/SF is a potent motility factor for epithelial cells leading to disruption and "scattering" of epithelial colonies in culture; it also induces DNA synthesis and cell division in several target cells (reviewed by Birchmeier *et al.*[1]). Thus, the activity of individual HGF/SF domains and selected domain combinations was studied both in colony scattering (Figure 6) and DNA synthesis assays (Figure 7) over a range of concentrations. Figure 6 shows representative results of MDCK colony scattering experiments (individual domains at 10^{-6} M, multidomain fragments at 10^{-7} M, full length HGF/SF at 10^{-10} M). Three individual kringle domains (K1, K2 and K4) caused dispersal of MDCK colonies (Figure 6(d), (e) and (g)) with variable potencies. The K1 domain (Figure 6(d)) was active at concentrations of 10^{-8} M or higher and, in contrast to other domains, showed a bell-shaped response, i.e. reduced activity at the highest concentration tested (10^{-5} M, data not shown). The K2 and K4 domains were active at concentrations of 10^{-6} and 10^{-5} M, respectively, whereas K3 was inactive at 10^{-6} M (Figure 6(f)) and only showed marginal activity at 10^{-5} M (data not shown). Met activation by individual HGF/SF kringles was specific because kringle domains from other proteins, for example kringle 4 from the HGF/SF homologue HGFl/MSP (K4*, Figure 6(l)), were inactive. In contrast to selected kringle domains, neither the N nor SP domains caused dispersal of MDCK colonies at 10^{-6} M (Figure 6(c) and (h)) or 10^{-5} M concentrations (data not shown).

The activity of individual HGF/SF domains and selected combinations on DNA synthesis in mouse MK cells is shown in Figure 7. K1 promoted DNA synthesis at concentrations from 10^{-8} M (data not shown) to 10^{-6} M; K2, K4 and the SP domains only showed modest activity at the highest concentrations; the N and K3 domains were inactive at all concentrations tested. Most multi-domain fragments were active in DNA synthesis assays: NK1 and NK2 produced strong stimulation but addition of the K3 domain (NK3) decreased activity and addition of both the K3 and K4 domains (NK4) abolished activity, in agreement with results from other laboratories.[39] K4-SP was more active than the K4 or SP domains alone but less active than NK1 and NK2 (Figure 7). Analysis of the phosphorylation status of ERK proteins, critical downstream effectors of the mitogenic response to HGF/SF, confirmed the activity, or lack of it, of different HGF/SF domains and their combinations (Figure 8).

Mapping the binding of individual HGF/SF domains on the β-propeller of Met

Met contains a furin site that is processed during secretion and yields a mature receptor consisting of disulphide-linked α (aa 25–307) and β-chains (aa 308–1390). As a result, the seven-blade β-propeller of Met (aa 25–514), the domain responsible for HGF/SF binding,[22,32] consists partly of Met α-chain (aa 25–307, blades 1–4) and partly of the β-chain (aa 308–514, blades 5–7). A co-crystal structure of the SP domain of HGF/SF in complex with a fragment of Met showed that the SP domain contacts an area of the β-propeller located within the α-chain,[22] but the binding sites of the other HGF/SF domains on the Met propeller are not well defined.

To map these sites, we carried out cross-linking experiments with Met741, a fragment of Met consisting of the β-propeller, the cystine-rich domain and the first two immunoglobulin-like domains.[32] This Met construct was used instead of Met567 because the α and β-chains of Met741 are readily distinguishable on SDS-polyacrylamide gels (42.5 and 61.8 kDa). After transfer to nitrocellulose membranes, the products of cross-linking were probed with anti-HGF/SF and/or anti-Met polyclonal antibodies, allowing assignment of individual bands to ligand, receptor or a complex of the two.

In the presence of BS^3, the α and β-chains of Met are rapidly cross-linked and, as expected, heparin dp12 has no effect on this process (Figure 9, left). For

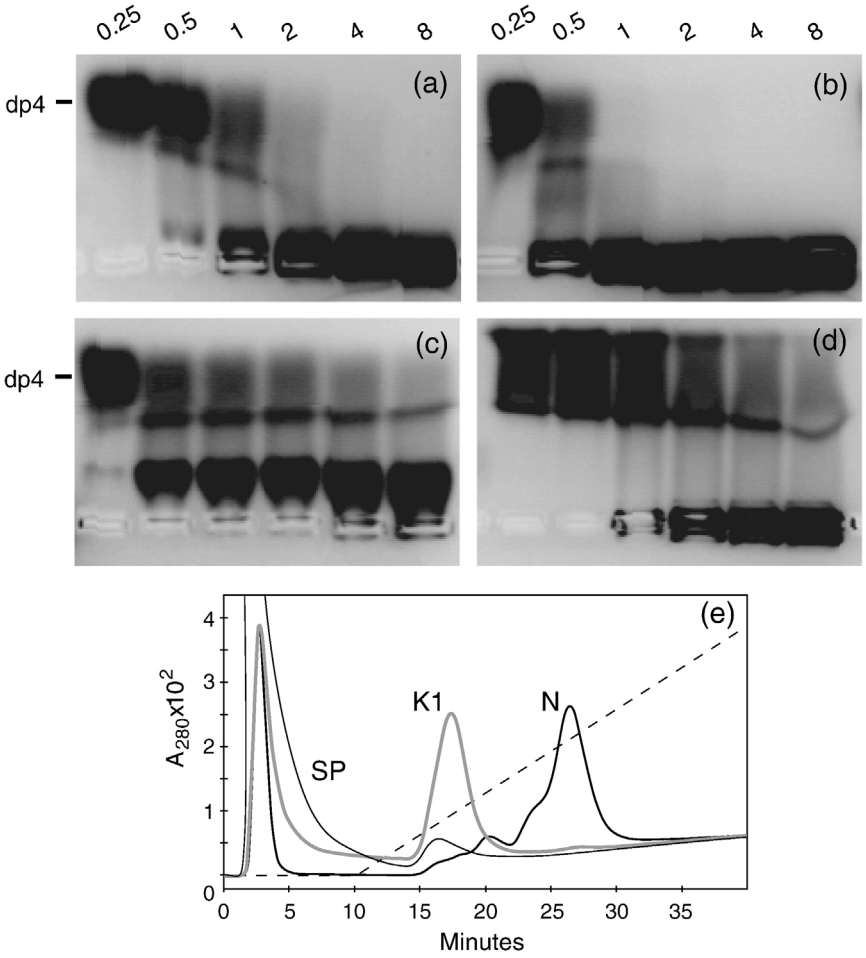

Figure 4. Titration of the binding of HGF/SF domains to heparin dp4 by gel mobility shift assay. (a) NK1, (b) N, (c) K1 and (d) SP domains in complex with AMAC-heparin dp4. Numbers across the top denote the ratio of protein to dp4. (e) Heparin-Sepharose column chromatography of the N, K1 and SP domains. Proteins were loaded onto the column in 25 mM phosphate, 75 mM NaCl (pH 7.0) and eluted with a NaCl gradient from 75 mM to 2 M (broken line).

the analysis of cross-linking of NK1 or the N, K1 and K2 domains to Met741 the following conditions were studied: (i) HGF/SF domain(s) + dp12 heparin + Met741 + BS^3, (ii) HGF/SF domain(s) + dp12 heparin + BS^3, (iii) HGF/SF domain(s) + Met741 + BS^3, (iv) HGF/SF domain(s) + BS^3 and, (v) HGF/SF domain(s) without BS^3. NK1 and the N, K1 and K2 domains readily yielded cross-linked products with Met741, i.e. bands reacting with both anti-HGF/SF and anti-Met antibodies. These included high molecular weight species in which both the α and β-chains of Met were covalently attached (and therefore ligand binding could not be assigned to either the α or β-chains), as well as bands that represented cross-linked products of the HGF/SF domains to the α or β-chains (Figure 9, arrows). The M_r of the products of such cross-linking (~87,000 for NK1, ~76,000 for the N domain and ~ 73,000 for K1 and K2) are consistent with the binding of these domains to the β-chain moiety of the β-propeller of Met but not with cross-linking to the α-chain. Heparin dp12 increased the amount of cross-linked products in the case of NK1 and the N domain (although the same protein-protein complexes were detectable even in the absence of heparin) but had no effect on the products of cross-linking of the K1 or K2 domains (Figure 9). These experiments thus establish that, in contrast to the SP domain, which binds the α-chain moiety of the Met β-propeller,[22] the N, K1 and K2 domains of HGF/SF bind the β-chain of the receptor.

Discussion

Mutagenesis and crystallographic data have clarified the mode of binding of the SP domain of HGF/SF to the Met receptor and its essential role in receptor activation.[21,22] These studies and the experiments reported here show that the SP domain, while displaying little or no agonistic activity itself, is essential for receptor activation by full length HGF/SF (compare the activity of NK4 and HGF/SF in Figures 6–8). In contrast, the NK1 fragment, which corresponds to the N-terminal region of the α-chain of HGF/SF and contains a separate binding site for Met, retains considerable agonistic activity as shown by studies with cells in

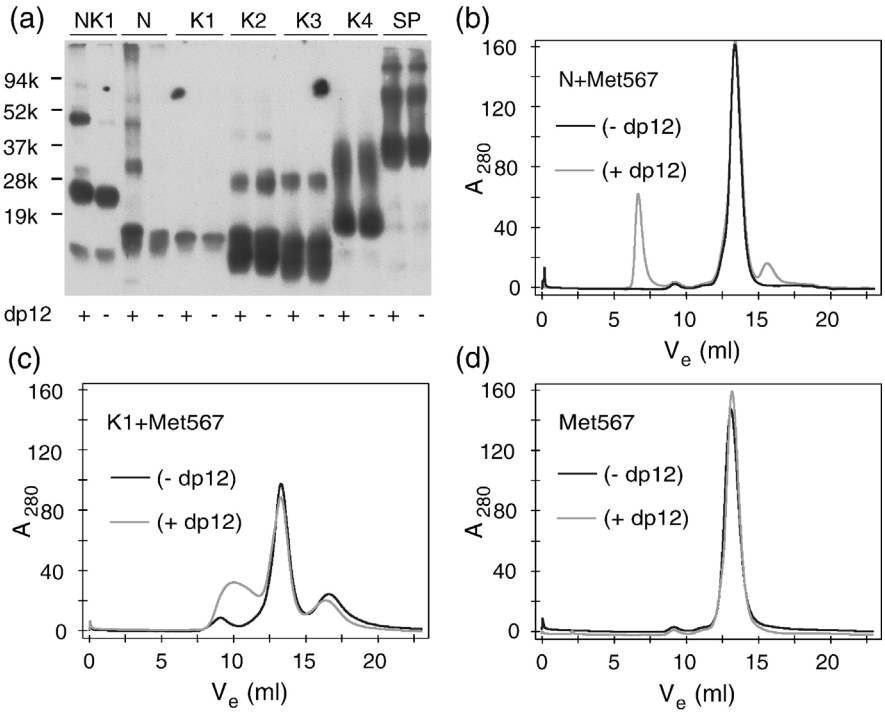

Figure 5. Effect of heparin on oligomerisation of individual HGF/SF domains and on Met binding by the N and K1 domain. (a) Cross-linking with BS^3 of 5 μM concentrations of protein domains in the absence of heparin or in the presence of equimolar dp12 heparin. The blot was probed with a polyclonal sheep anti-HGF/SF antiserum (1W53). (b) and (c) Gel filtration of N domain/Met567 mixtures (b), or K1 domain/Met567 mixtures (c) in the absence or presence of heparin dp12. Arrows indicate ligand-heparin-receptor complexes as confirmed by slot blot analysis with anti-HGF/SF or anti-Met antibodies. The main peak contains free Met567 and small amounts of 1:1 ligand receptor complexes by slot blot. (d) Elution profiles of Met567 alone in the absence or presence of dp12 heparin.

culture ([30,33] and Figures 6–8 here) and *in vivo*.[40,41] These results have produced a "Met activation puzzle", namely: (i) two HGF/SF domains (N and K1) are sufficient for receptor binding and activation, (ii) addition of one (NK3) or two further kringle domains (NK4) does not impair receptor binding but reduces or abolishes Met activation and, (iii) addition of the C-terminal, SP domain reconstitutes full agonistic activity (Figures 6–8). While the solution of the puzzle will require multiple crystal structures of Met in complex with the different ligands, the present study offers several novel clues to the structure/function of individual HGF/SF domains and the mechanism underlying Met activation, and these are discussed below.

The high-affinity Met binding site of HGF/SF

A construct of the N domain of HGF/SF expressed in *Escherichia coli* and refolded from inclusion bodies[42] has been used extensively in order to obtain an NMR structure[43] and in order to define its heparin binding.[44] Here we show that the individual N and K1 domains of HGF/SF expressed in *P. pastoris* bind Met with comparable affinities (Figure 2(b) and (c)) and that the "high affinity binding site" of the α-chain of HGF/SF thus consists of two sites (or half sites) located in the N and K1 domains. The K2 and K4 domains bind Met with lower affinity (Figure 2(d) and (f)) and further experiments will be required in order to clarify whether the partial agonistic activity displayed by these domains reflects binding to specific K2 and K4 sites on the Met β-propeller or a lower affinity occupancy of the K1 binding site. The K3 domain displays little or no Met binding and activation (Figures 6–8) and the main role of this domain may consist of providing the correct spacing between the NK1 and SP receptor binding sites.

Domains responsible for HGF/SF binding to GAG co-receptors

Our study also provides new data on the interaction between HGF/SF and co-receptor molecules, namely HS and DS. The unusual ability of HGF/SF to interact with DS with high affinity has been confirmed here and mapped to the N domain (Figure 3(a)), the domain containing the high-affinity heparin-binding site.[30,37] This suggests that the heparin-binding site of the N domain defined crystallographically (R_{73}, K_{58}, K_{60}, T_{61}, K_{62} and R_{76})[30] and by NMR titration studies[44] may also mediate binding to DS and, indeed, preliminary NMR titrations indicate a substantial overlap of the HS and DS-binding sites within NK1 (D. Uhrin and M.L., unpublished data). Yet most other heparin/HS-binding proteins fail to bind DS with good affinity,[25,26] implying some unique feature of the site present in the N domain of HGF/SF which awaits

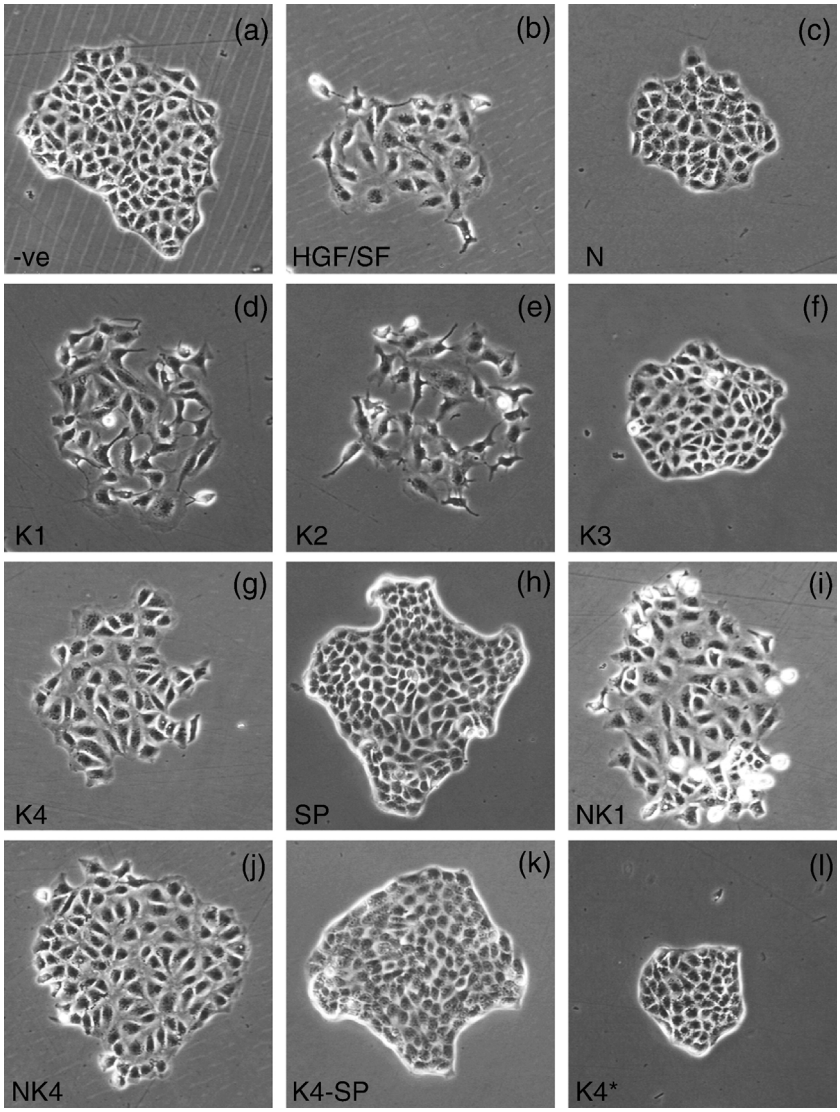

Figure 6. MDCK colony scatter assay in the presence of single domains or multidomain fragments of HGF/SF ((a)–(k)) or HGFl/MSP (l). (a) Control, (b) 10^{-10} M HGF/SF, (c) 10^{-6} M N domain, (d) 10^{-6} M K1 domain, (e) 10^{-6} M K2 domain, (f) 10^{-6} M K3 domain, (g) 10^{-6} M K4 domain, (h) 10^{-6} M SP domain, (i) 10^{-7} M NK1, (j) 10^{-7} M NK4, (k) 10^{-7} M K4-SP, (l) 10^{-6} M K4 domain from HGFl/MSP (magnification: 100×).

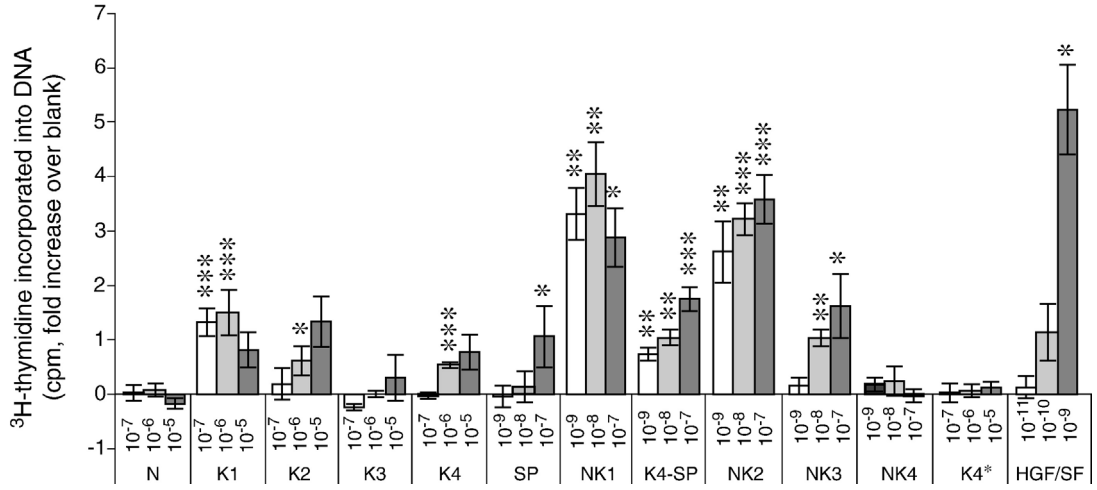

Figure 7. Effects of HGF/SF domains or multidomain fragments on [^{3}H]thymidine incorporation into DNA in MK cells. The proteins were incubated with cells at the concentrations shown for 18 h in the presence of [^{3}H]thymidine. K4* is a control kringle domain (K4 from HGFl/MSP). Data are expressed as fold increases over the basal rate of incorporation (no protein) in order to normalise data from different experiments. Error bars denote the standard deviation of readings around the mean. * = $P < 0.05$. ** = $P < 0.01$. *** = $P < 0.001$.

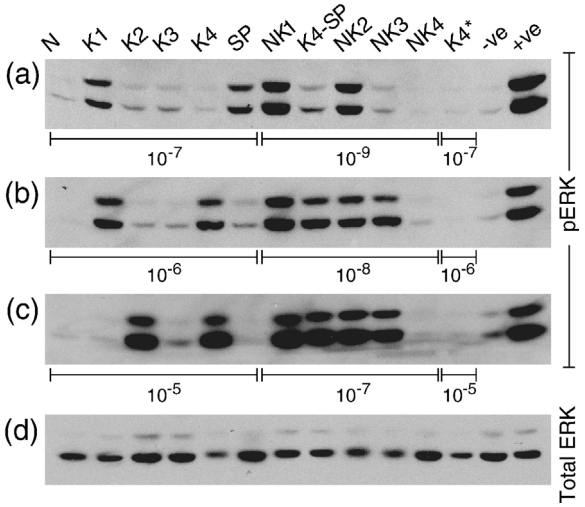

Figure 8. Activation of ERK by HGF/SF domains or multidomain fragments. The Figure shows Western blots of phospho-ERK and a representative blot of total ERK after treatment with HGF/SF domains at the concentrations shown. K4* is a control kringle domain (K4 of HGFl/MSP). Cultures used as negative controls received no addition, whereas positive controls were treated with 3×10^{-9} M HGF/SF.

clarification. Two additional heparin-binding sites have been mapped in the present study to the K1 and SP domains (Figure 3(b)). The site on the SP domain is of low affinity, as shown by chromatography on immobilised heparin (Figure 4(e)) and the poor binding of short, heparin dp4 fragments (Figure 4(d)). The biological relevance of this interaction (if any) therefore remains to be defined. The site on the K1 domain, in contrast, binds heparin dp4 in gel shift assays (Figure 4(c)), induces Met oligomerisation in gel filtration experiments (Figure 5(c)) and provides an explanation for the earlier observation that the agonistic activity of K1 requires heparin or HS.[34] The failure of the isolated K2 domain to bind heparin was surprising, given the results of earlier domain deletion[36] and point mutagenesis experiments[37] and it is conceivable that heparin-binding by K2 requires the presence of the N and K1 domains and/or a restrained orientation of the K2 domain itself caused by the inter-kringle K2-K3 disulphide bond.

Mapping the binding of individual domains on the Met β-propeller

Our cross-linking studies have clarified further the way in which HGF/SF binds Met. The β-propeller of Met (shown schematically in Figure 10(a) with the α-chain in white and the β-chain in grey) contains a long insertion in blade 5 which packs against blade 4.[22] As a result, the contribution of the β-chain to the side face of the domain is greater than for the top and bottom faces (Figure 10(a)). The co-crystal structure published by Stamos and colleagues showed that the SP domain of HGF/SF interacts with the bottom face of the Met propeller in a region corresponding to the α-chain[22] (Figure 10(b)). On the other hand, low-resolution models of a complex of full length HGF/SF and the Met ectodomain, based on 3D reconstructions from cryo-EM and

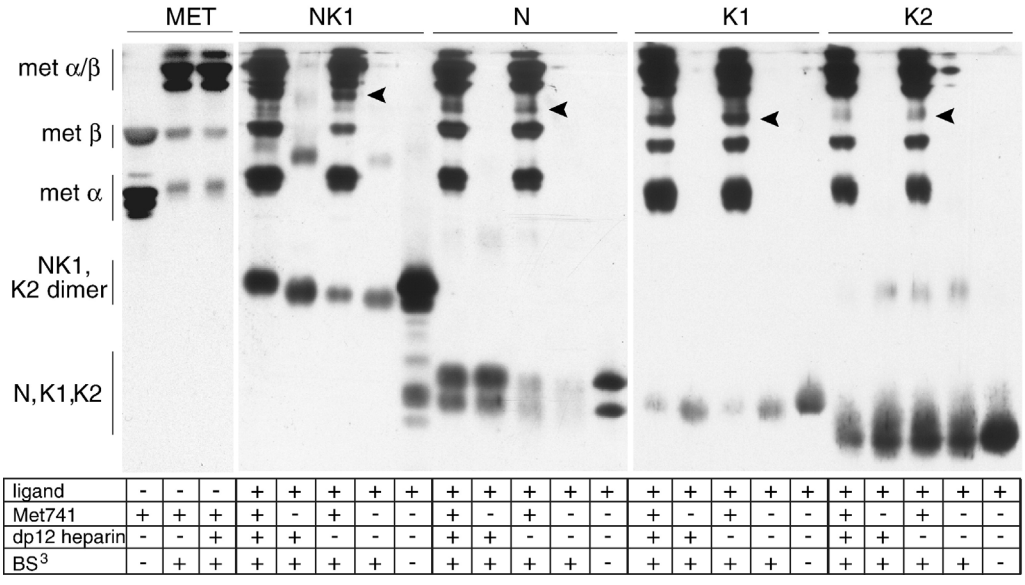

Figure 9. Cross-linking of NK1 or the N, K1 and K2 domains of HGF/SF to Met741. The first three lanes on the left show cross-linking of the Met α and β-chains by BS^3 in the absence or presence of dp12 heparin. For each HGF/SF domain the following conditions are shown: −HGF/SF domain + dp12 heparin + Met 741 + BS^3, −HGF/SF domain + dp12 heparin + BS^3, −HGF/SF domain + Met 741 + BS^3, −HGF/SF domain + BS^3 and HGF/SF domain alone. Proteins were at a concentration of 5 μM and BS^3 at 500 μM. Blots were probed with a mixture of polyclonal sheep anti-HGF/SF and mouse anti-Met antibodies and arrowheads show bands representing the products of cross-linking between the HGF/SF domains and the Met β-chain.

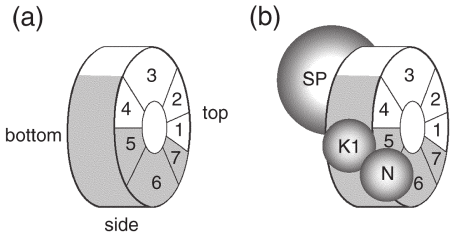

Figure 10. Binding of HGF/SF domains to the Met β-propeller. (a) Schematic representation of the β-propeller of Met. The α chain is in white, the β chain in grey; numbers represent individual blades and labels indicate the three faces of the Met domain discussed in the text. (b) Binding of the SP, N and K1 domains of HGF/SF to the Met β-propeller. The position of the SP domain is from the crystal structure described by Stamos *et al.*,[22] the positions of the N and K1 domains are based on low resolution models from cryo-EM and small angle X-ray scattering data[23] as well as the results of the experiments reported here (Figure 9).

small angle X-ray scattering (SAXS), have suggested that the binding sites for the N and K1 domains may be located on the top and side faces of the Met β-propeller[23] (Figure 10(b)). The cross-linking experiments reported here, which map the binding sites for the N and K1 domains in the area corresponding to the Met β chain, support the conclusions of the cryo-EM and SAXS experiments. The model shown in Figure 10(b) is testable in at least two ways: (i) although HGF/SF and Met complexes have failed as yet to yield crystals, co-crystallisation experiments with the isolated SP domain have been successful.[22] The binding affinities of the N and K1 domains are comparable to the SP domain (Figure 2 and Results); hence co-crystallisation experiments with isolated N or K1 domains are a viable option, (ii) recent developments in mass spectrometric analysis of cross-linked proteins and protein complexes[45] offer a powerful and independent route for fine mapping of the binding sites for the N and K1 domains on Met.

Protein engineering of HGF/SF domains for receptor antagonistic activity

Although the N and K1 domains bind Met with comparable affinities (Figure 2), the two domains differ radically in their ability to bind Met (Figure 5) and induce receptor activation (Figures 6–8). The present study thus confirms earlier observations by Rubin and colleagues demonstrating that while the K1 domain retains agonistic activity the N domain does not.[34] Here we have demonstrated that the lack of agonistic activity of the N domain is not due to failure to bind Met (Figure 2(b)) or to dimerise in the presence of heparin/HS co-receptors (Figure 5(a)). The N domain can bind Met in the absence of heparin (Figures 2 and 9) and does produce very large (M_r>1,300,000) ligand-receptor complexes in the presence of heparin (Figure 5(b)), in contrast to the biologically active K1 domain, which forms a complex of ~350,000 with Met567 in the presence of heparin (Figure 5(c)). The exact stoichiometry of this K1-Met complex and the mechanism by which heparin causes oligomerisation await to be defined, but our study suggests that this complex is an active, signalling one that deserves further structural analysis.

The results of the above experiments with the N and K1 domains have direct implications for protein engineering of Met antagonists. The ability of the N domain to bind Met while failing to lead to receptor activation raises the prospect that an engineered form of this domain with increased affinity for Met receptor may yield an effective antagonist for cancer therapy. NK4 has been extensively characterised as a Met antagonist[39] but the protein is difficult to produce in the quantities needed for protein therapy and may require a gene therapy approach. By contrast, the N domain can be produced in large quantities in yeast (this work) or in bacteria.[42] In an alternative approach we propose that the K1 domain, which binds Met in the absence of heparin (Figures 2 and 9), but requires heparin for Met oligomerisation (Figure 5(c)) and for biological activity,[34] may be converted into a receptor antagonist by destroying its heparin-binding site. These new strategies to protein engineering of Met antagonists can now be explored on the basis of the findings of the present study.

Materials and Methods

Cloning of HGF/SF fragments

Fragments were cloned from cDNA of full length human HGF/SF (or HGFI/MSP for the control kringle of HGFI/MSP kringle 4, referred to as K4*) into pPIC9K with the addition of a C-terminal hexahistidine tag for expression in *P. pastoris*, or pA71d also with a hexahistidine tag for expression in mammalian systems. Before cloning into pA71d, constructs were subcloned into a pBluescriptIISK(+) vector to introduce an immunoglobulin leader sequence. The Met567 and Met741 construct design and protein expression have been described.[32] All constructs were sequenced and found to match the sequence of hgf_human in the UniProt database, except K3 (V374A).

Expression and screening of HGF/SF fragments

Transformation of GS115 *P. pastoris* was performed by linearising 10 μg pPIC9K containing HGF/SF cDNA fragment insert with SalI then following the protocol for spheroplasting using a Spheroplast kit (Invitrogen). Transformants were initially selected based on their ability to grow in the absence of histidine on regeneration dextrose plates. Subsequent screening was performed by selection of G418-resistant colonies. Clones were screened by following the Invitrogen manual for protein expression in *P. pastoris* and then probing blots of supernatant with 1:1000 sheep anti-HGF/SF (1W53) in 5% (w/v) milk/PBS. Secondary antibody was 1:1000 rabbit anti-sheep (Dako) and the blot was developed using diaminobenzidine. Once the best expressing clones had been selected,

expressions were scaled up to several litres in conical flasks. Filtered supernatant was then dialysed/concentrated using a crossflow machine (Flowtech Inc) containing a 5 kDa/10 kDa Hydrosart slice cartridge (Sartorius) chilled to 10 °C. The SP domain was expressed as K4-SP by transfection of NS0 cells by electroporation. Stable transfectants were then selected based on hygromycin resistance conferred by the pA71d vector. Expression was scaled up in DMEM + 2.5% (v/v) foetal calf serum (FCS) in 2 l roller bottles incubated at 37 °C. Harvesting of 90% of the culture was effected twice a week and was then filtered/crossflowed through a 10 kDa Hydrosart slice cartridge.

Purification

Nickel-affinity chromatography (Histrap; Amersham) was used as an initial purification step for all constructs using 20 mM phosphate, 0.5 M NaCl (pH 7.4), with a gradient of imidazole up to 500 mM. Eluted fractions containing Met567 were applied to a heparin-affinity column (Hitrap heparin; Amersham) to remove any single-chain Met (i.e. uncleaved by furin) that remained in the sample. The resulting homogeneous two-chain Met fraction was then further purified using gel filtration (Superdex 200 16/60; Amersham) with 50 mM Tris (pH 7.4), 150 mM NaCl. The single domains were further purified after HisTrap using gel filtration (Superdex 75 10:30, Amersham) into 50 mM Tris, 150 mM NaCl (pH 7.4) (K1, K2, K3, K4), 20 mM phosphate, 0.5 M NaCl (pH 7.4) (N), or 50 mM Mes, 250 mM NaCl (pH 6.0_ (K4-SP). To increase stability, 50 mM L-arginine/glutamic acid[46] was added to all buffers during purification of N, K1 and K4-SP. NK1 was purified as described.[30] The SP domain was cleaved from K4-SP by incubating with 10 μg porcine pancreatic elastase per 1 mg K4-SP at room temperature for 16 h. HisTrap was used to separate SP and residual K4-SP from K4 based on the C-terminal hexahistidine tag. The SP domain was further purified by gel filtration with 50 mM Mes, 250 mM NaCl, 50 mM L-arginine/glutamic acid (pH 6.0).

Analytical ultracentrifugation

Protein samples were diluted to 20 μM then loaded into the AUC cell and centrifuged at 60,000 rpm at 20 °C in an Optima XL-A ultracentrifuge (Beckman) with an An60 Ti rotor using only a single cell. Absorbance scans at 280 nm were measured as fast as possible (i.e. approximately once every 1.5 min). Data were analysed using the d*c*/d*t* method[47] by the computer program DC/DT+.[48] Partial specific volumes, solvent density and viscosity were calculated using SEDNTERP (RASMB).[49] Sets of ten scans were used to generate a plot of $g(s^*)$ (material sedimenting between s^* and $s^*+\delta s^*$) against $s^*_{20,w}$ (apparent sedimentation coefficient). The raw data were also fitted for either a single, or two, component(s) using DC/DT+, to see which model fitted better and to determine the best value(s) for $s^*_{20,w}$. Quality of fitting was judged from plots of the residuals between calculated and experimental data at each scanned radius.

Cell motility assays

Madin-Darby canine kidney (MDCK) cells were used to seed 6-well tissue culture plates (Falcon) at a density of 2500 cells per well. Cells were then incubated in DMEM (from Clare Hall, CRUK laboratories) + 5% FCS (Gibco) at 37 °C/5% CO_2 for 48 h and next treated with proteins diluted to various concentrations in E4 + 5% FCS and incubated for 20 h. Photographs were taken using a Hamamatsu camera with a phase contrast lens at 100 times magnification.

DNA synthesis assay

Mouse keratinocyte (MK) cells were used to seed 24-well tissue culture plates (Falcon) at a density of 2500 cells per well. Cells were incubated in keratinocyte medium (Gibco) supplemented with 50 μg/ml bovine pituitary extract (BPE), 5 ng/ml recombinant epidermal growth factor (EGF) and penicillin/streptomycin (complete KM) at 37 °C/5% CO_2. When 100% confluence was reached, cells were washed twice with PBS, then incubated in serum-free keratinocyte medium (SFKM) for 24 h. SFKM (1 ml) containing 1 mg/ml BSA, 75 μg/ml thymidine, 1 μCi/ml [^{3}H]methylthymidine was then added to each well followed by the addition of proteins diluted in SFKM. The cells were then incubated for 18 h to allow incorporation of [^{3}H]methylthymidine into newly synthesised DNA. Free [^{3}H]methylthymidine was removed by washing cells with 5% TCA, then cells were lysed with 0.2 M NaOH. Cell lysate was diluted 1:25 into scintillation fluid (Fluorosafe from BDH) and incubated at room temperature in the dark for 3 h before counting each vial for 1 min.

Western blots

To test for the effect of HGF/SF fragments on cell signalling, MK cells were used to seed six-well tissue culture plates (Falcon) and were grown in complete KM for seven days (approximately 80% confluent) before synchronisation with SFKM for 24 h. Proteins diluted in SFKM were added to each plate and incubated at 37 °C for 5 min and were then lysed with 200 μl of lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40, 1 mM orthovanadate, 2 mM EDTA, 5 mM NaF) on ice for 30 min. ERK MAP kinase was detected using rabbit polyclonal anti-ERK1/2 (Promega) at a dilution of 1:10,000 and mouse monoclonal anti-diphosphorylated ERK1/2 (Sigma-Aldrich) at a dilution of 1:20,000. Secondary antibodies were goat anti-rabbit HRP and rabbit anti-mouse HRP (Dako) and both were used at a dilution of 1:2000. Bands were visualized by incubating probed membranes in 100 mM Tris, 1.25 mM luminol (Sigma-Aldrich), 0.2 mM *p*-coumaric acid (Sigma-Aldrich), 0.01% H_2O_2.

Surface plasmon resonance

To immobilise Met567H, a single flow cell of a CM5 chip was equilibrated with 20 mM phosphate (pH 7.4), 150 mM NaCl, 50 μM EDTA, 0.005% Surfactant P20. The chip surface was then activated using the CM5 amine coupling immobilization method in the BIACore Control software. This injects 100 μl of 0.2 M EDC mixed with 100 μl 0.05 M NHS to give reactive succinimide ester groups. Met567H (400 nM) diluted in 10 mM sodium acetate was then injected, which spontaneously binds *via* amine groups to the surface succinimide esters until a ΔRU of 1500 is reached. Remaining active groups are then neutralised by injection of 70 μl of 1 M ethanolamine (pH 8.5). As a negative control, another flow cell of the CM5 chip was

treated with the same program, but without protein. The chip was then re-equilibrated with HBS-EP (10 mM Hepes (pH 7.4), 150 mM NaCl, 50 μM EDTA, 0.005% Surfactant P20) + 0.2 mg/ml BSA and then a range of protein concentrations (diluted in HBS-EP + BSA) were injected at 20 μl/min for 60 min followed by a 300 s dissociation time. The chip was regenerated using HBS-EP with 1 M NaCl.

Gel mobility shift assay (GMSA)

Heparin (Innohep; Leo Pharma, Princes Risborough, UK) and porcine intestinal mucosal dermatan sulphate (Celsus Laboratories Inc., Cincinatti, OH, USA) were partially depolymerised by specific enzymatic action and fractionated into specific oligosaccharide sizes by gel filtration chromatography, as described by Goger *et al.*[50] and Lyon *et al.*,[25] respectively. Oligosaccharides were labelled with 2-aminoacridone (AMAC) as described by Lyon *et al.*[29] Gel mobility shift assay was performed as described by Lyon *et al.*,[29] except that 1% (w/v) agarose gels were used on a Bio-Rad Sub-Cell GT horizontal agarose electrophoresis system, substituting for the previously described 6% polyacrylamide gels, but using the same buffer systems. Protein and AMAC-oligosaccharides were pre-incubated in volumes of 10 μl, loaded into the wells and electrophoresed for 8 min at 200 V. Subsequent visualisation was as described.[29]

Heparin-Sepharose chromatography

Approximately 0.8 ml bed volume of heparin-Sepharose (Sigma) was packed into an empty 5 mm diameter HR 5/5 column (Amersham Biosciences) and run under HPLC conditions. After equilibration with 25 mM sodium phosphate, 75 mM NaCl (pH 7.0), protein samples were loaded (100 μl) and washed with the same solution at 0.5 ml/min, before application of a linear gradient of 75 mM – 2 M NaCl in phosphate buffer. The gradient was generated using an Agilent 1100 series quaternary HPLC pump and the eluate monitored using in-line UV detection at 214 nm.

Gel filtration chromatography

Samples of the N or K1 domain (50 μM) were incubated for 2 h at room temperature with equimolar concentrations of Met567 and/or heparin dp12 in a final volume of 100 μl of 25 mM Tris-HCl (pH 7.4), 0.1 M NaCl before they were applied to a Superdex 200 (HR 10/30) column (Amersham Biosciences). The column was eluted at 0.5 ml/min in the same buffer and the eluate monitored at 280 nm (for detection of protein or protein-heparin complexes) or at 228 nm (for detection of heparin alone).

Cross-linking

NK1 or individual HGF/SF fragments (5 μM) were incubated in the presence or absence of equimolar dp12 heparin and/or Met741 for 2 h at room temperature in 20 mM phosphate (pH 7.4), 100 mM NaCl. BS^3 was then added to a final concentration of 500 μM and incubated at room temperature for 30 min. The cross-linking reaction was quenched by adding Laemmli dye (leading to a final Tris concentration of 85 mM).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.12.061

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