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Scatter factor and hepatocyte growth factor are indistinguishable ligands for the *MET* receptor

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Scatter Factor (SF) is a fibroblast-secreted protein which promotes motility and matrix invasion of epithelial cells. Hepatocyte Growth Factor (HGF) is a powerful mitogen for hepatocytes and other epithelial tissues. SF and HGF, purified according to their respective biological activities, were interchangeable and equally effective in assays for cell growth, motility and invasion. Both bound with identical affinities to the same sites in target cells. The receptor for SF and HGF was identified as the product of the *MET* oncogene by: (i) ligand binding and coprecipitation in immunocomplexes; (ii) chemical cross-linking to the Met β subunit; (iii) transfer of binding activity in insect cells by a baculovirus carrying the *MET* cDNA; (iv) ligand-induced tyrosine phosphorylation of the Met β subunit. SF and HGF cDNA clones from human fibroblasts, placenta and liver had virtually identical sequences. We conclude that the same molecule (SF/HGF) acts as a growth or motility factor through a single receptor in different target cells.

Key words: growth factor receptor/hepatocyte growth factor/*MET* oncogene/scatter factor/tyrosine kinase

Introduction

Scatter Factor (SF) is a secretory product of fibroblasts which dissociates epithelial cells increasing their motility and invasiveness (Stoker *et al.*,1987; Rosen *et al.*, 1990; Weidner *et al.*, 1990). It was reported to be chemotactic and not mitogenic for target cells (Gherardi *et al.*, 1989). SF might be involved in the progression of carcinoma cells to a more malignant invasive phenotype (Weidner *et al.*, 1990) and might play a role in epithelial–mesenchymal transitions during early embryonic development (Stern *et al.*, 1990).

Hepatocyte Growth Factor (HGF) is a powerful mitogen for hepatocytes in primary cultures. It was isolated from several sources including rat platelets (Nakamura *et al.*, 1986), serum of human patients with hepatic failure (Gohda *et al.*, 1988), and rabbit serum (Zarnegar and Michalopoulos, 1989). HGF is considered a major mediator of liver regeneration *in vivo* (Michalopoulos, 1990). Recently, HGF was shown to stimulate the growth of other epithelial tissues, such as kidney tubular epithelium and keratinocytes (Kan *et al.*, 1991), endothelial cells and melanocytes (Rubin *et al.*, 1991).

While the biological activities of SF and HGF are apparently unrelated, purification of the molecules revealed a surprising degree of structural similarity. Both HGF and SF are disulphide-linked heterodimers of a heavy (α) subunit of 55–65 kDa and a light (β) subunit of 32 or 36 kDa. The two M_r of the β subunit of SF were ascribed to differences in glycosylation (Weidner *et al.*, 1990). The α and β subunits of HGF originate from proteolytic cleavage of a single 92 kDa precursor, as indicated by the sequence of cloned human and rat HGF cDNAs (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989; Tashiro *et al.*, 1990). When the primary sequence of several tryptic peptides derived from purified SF was compared to the deduced amino acid sequence of HGF, all the identified residues could be matched (Gherardi and Stoker, 1990; Weidner *et al.*, 1990; 1991).

Previous work by Naldini *et al.* (1991a) and that of another laboratory (Bottaro *et al.*, 1991) has recently suggested that the HGF receptor is the product of the *MET* oncogene, a transmembrane protein endowed with tyrosine kinase activity (Cooper *et al.*, 1984; Park *et al.*, 1986, 1987). The structure of the Met protein has been investigated in a cell line (GTL16), where the gene is amplified and overexpressed (Giordano *et al.*, 1989a). The protein is a 190 kDa heterodimer ($p190^{MET}$) made of a 50 kDa subunit (α) disulphide-linked to a 145 kDa subunit (β). The molecule is synthesized as a single-chain 170 kDa precursor, which undergoes co-translational glycosylation. Disulphide rearrangements and proteolytic cleavage lead to the mature two-chain 190 kDa heterodimer (Giordano *et al.* 1989b). The α chain and the N-terminal portion of the β chain of the mature protein are exposed at the cell surface (Giordano *et al.*, 1988). The C-terminal portion of the β chain is cytoplasmic and includes a tyrosine kinase domain (Dean *et al.*, 1985; Tempest *et al.*, 1986; Gonzatti *et al.*, 1988) and phosphorylation sites involved in regulation of its activity (Ferracini *et al.*, 1991). The kinase activity is positively regulated by autophosphorylation on tyrosine (Naldini *et al.*, 1991b), and it is negatively regulated by protein kinase-C activation (Gandino *et al.*, 1990) or transient increases of intracellular Ca^{2+} concentrations (Gandino *et al.*, 1991). Stimulation of the tyrosine phosphorylation of the β subunit of the Met protein after exposure to HGF was observed both in intact cells and *in vitro* with partially purified Met protein (Bottaro et al., 1991; Naldini *et al.*, 1991a). Chemical cross-linking to the Met protein of a molecule smaller than HGF (M_r 28 kDa) but with similar binding properties was also reported (Bottaro *et al.*, 1991).

In this work we have investigated the structural and functional relationships between SF and HGF. Nucleotide sequence analysis of cDNA clones from fibroblasts, placenta

and liver showed that SF and HGF are indistinguishable. The two molecules were interchangeable and equally effective in inducing the different biological responses. Moreover, SF and HGF competed with identical affinity for the same p190^{MET} receptor, triggering its tyrosine kinase activity in different target cells.

Results

SF and HGF are functionally interchangeable in bioassays

Human SF was purified to homogeneity from medium conditioned by MRC5 embryo lung fibroblasts as described elsewhere (Weidner *et al.*, 1990). Bioactivity was measured by the scatter assay on Madin–Darby canine kidney (MDCK) epithelial cells (Stocker *et al.*, 1987). Pure SF had a specific activity of ~0.5 units/ng. Human HGF was purified to homogeneity from human placenta as previously described (Zarnegar and Michalopoulos, 1989; Zarnegar *et al.*, 1989). Bioactivity was measured by induction of DNA synthesis in hepatocytes in primary culture. A full mitogenic response was scored when 85% of cells entered S phase and was obtained with 10 ng/ml of the pure HGF preparation. The concentration and purity of both factor preparations were assessed by SDS–PAGE followed by silver staining and by SDS–PAGE and autoradiography after ^{125}I-labelling (see Figure 3).

The pure factor preparations were compared in the respective bioassays. SF was as potent a mitogen as HGF in a hepatocyte growth assay. Both factors induced a several-fold increase in [^{3}H]thymidine incorporation into rat hepatocytes in primary cultures (Figure 1). When tested on

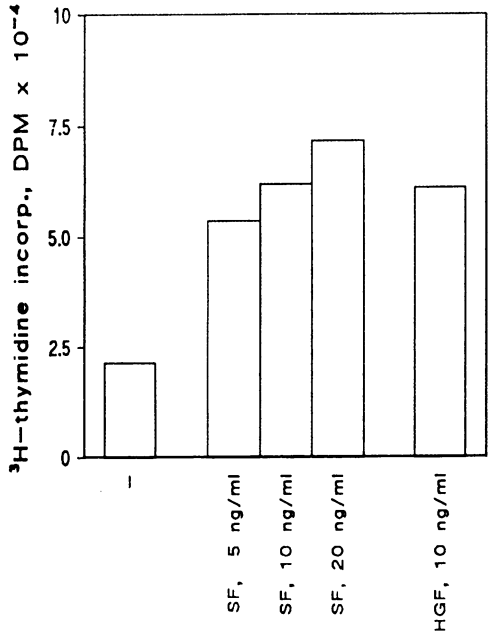

Fig. 1. SF stimulates the DNA synthesis of rat hepatocytes in primary culture as effectively as HGF. Rat hepatocytes were isolated by liver perfusion with collagenase and cultured on six-well collagen-coated plates in medium supplemented with insulin as described (Zarnegar *et al.*, 1991). The cultures were incubated with the indicated concentrations of factor for 24 h, then [^{3}H]thymidine (1 μCi/ml) was added in fresh medium for a further 18 h. DNA synthesis was evaluated by [^{3}H]thymidine incorporation into TCA-precipitable material. The data shown are the average of replicate determinations from a typical experiment.

the A459 human lung carcinoma cell line, HGF induced cell dissociation as effectively as SF (Figure 2). Moreover, in an assay of invasion into collagen matrices, SF and HGF were equally effective in promoting a 10- to 20-fold increase in the number of cells entering into the matrices. This effect was observed in a panel of human cell lines including A549 lung carcinoma cells, HS766T and Capan 1 pancreas carcinoma cells (Table I).

SF and HGF are thus functionally interchangeable and equally effective in bioassays of cell growth, motility and matrix invasion.

SF and HGF bind with identical affinities to the same sites at the cell surface

Purified SF was radiolabelled with ^{125}I and Iodogen (Pierce) to a specific activity of ~8×10^7 c.p.m./μg (5700 Ci/mmole). The radiolabelled ligand retained biological activity, albeit reduced 4- to 5-fold from the level of the native molecule as assessed by hepatocyte growth (data not shown). When analysed in SDS–PAGE [^{125}I]SF migrated as a diffuse band of M_r ~74–78 kDa in non-reducing conditions. After reduction of disulphide bonds a small fraction of [^{125}I]SF migrated as a M_r 90 kDa unprocessed precursor (preSF) while most of it was split into its two subunits, α of 55–65 kDa and β_1 and β_2 of 36 and 32 kDa, respectively (Figure 3).

[^{125}I]SF bound specifically to the cell surface of A549 carcinoma cell lines. Equilibrium binding was measured after incubation of cell monolayers with sub-nanomolar concentration of [^{125}I]SF in the presence of increasing concentrations of unlabelled SF or HGF at 4°C for 3 h. After extensive washing, the monolayers were extracted with 1% Triton X-100 and counted. The ligand displacement curves obtained with SF and HGF are shown in Figure 4. The two curves overlapped entirely and indicated the presence of two classes of binding site, of higher and lower affinity. The high capacity, lower affinity binding site (K_d ~2 nM) was most likely due to heparansulphate proteoglycans associated with the cell membrane and/or the extracellular matrix. The two classes of binding sites were in fact differentially eluted by heparin. After equilibrium binding as above, the monolayers were incubated for 5 min at 4°C with excess heparin. The eluate was collected and counted. While the lower affinity site was eluted by excess heparin (Figure 4), the higher affinity site (K_d ~0.2 nM) quantitatively retained the bound ligand and was extracted only by the detergent.

A ligand displacement curve similar to that shown above for A549 cells was obtained using GTL16 cells. Two classes of binding site with affinities similar to those found in A549 cells were observed. However, the binding capacity of the higher affinity site was much larger than in A549 cells (data not shown).

SF and HGF thus compete with equal affinities for the same binding sites on A549 and GTL16 target cells.

The high affinity receptor for SF and HGF is p190^{MET}

The overexpression of the high affinity binding site in GTL16 cells was exploited for the molecular identification of the SF and HGF receptor.

After equilibrium binding of HGF at 4°C, GTL16 monolayers were eluted with heparin, surface-labelled with [^{125}I]iodine and lactoperoxidase and extracted with detergent. The *MET* oncogene product was

immunoprecipitated with an antiserum directed against the carboxy-terminus of the protein (anti-Met-COOH). As shown in Figure 5A, HGF was coprecipitated with the Met protein. The identity of the coprecipitated molecule with HGF was confirmed by its migration pattern in reducing conditions (data not shown). Neither the Met protein nor HGF were present in control immunoprecipitates made with preimmune serum (not shown). Similar results were also obtained with SF (see Figure 6).

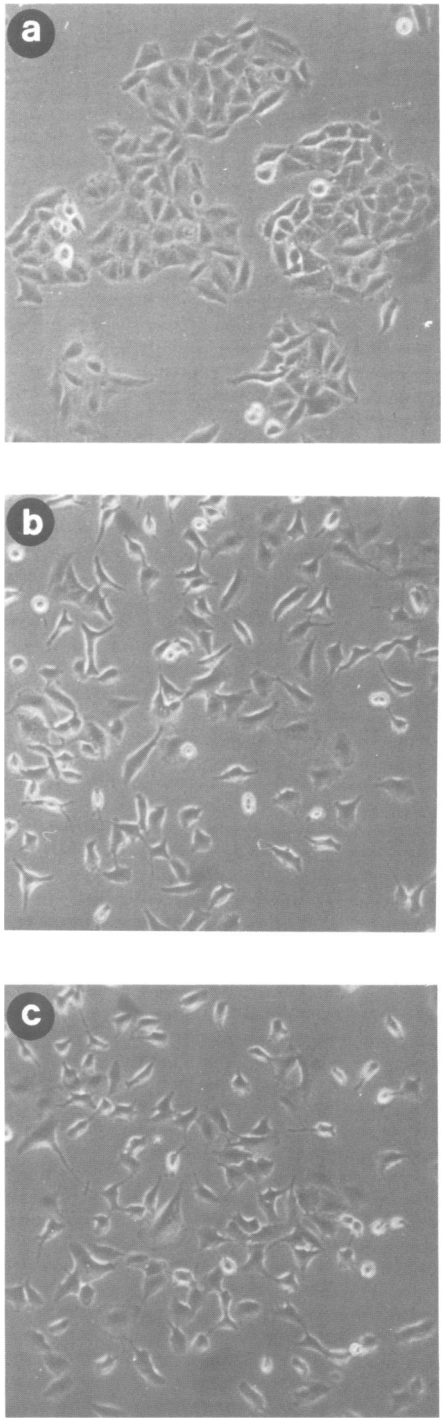

Fig. 2. HGF dissociates colonies of A549 epithelial cells as effectively as SF. A549 human lung carcinoma cells were incubated overnight without (**a**) or with 10 ng/ml of purified SF (**b**) or HGF (**c**).

The presence of high affinity binding sites for SF and HGF in immunoprecipitates of the Met protein was further proved by direct binding of [^{125}I]SF. Immunocomplexes obtained from GTL16 cell extracts using monoclonal antibodies against a carboxy-terminal peptide of the Met protein, covalently coupled to protein A–Sepharose, bound specifically [^{125}I]SF. While a 20-fold excess of unlabelled SF or HGF displaced the tracer from the immunocomplexes, excess heparin was ineffective (Figure 5B). No binding activity was precipitated with protein A–Sepharose charged with control antibodies or from cells not expressing the *MET* oncogene (not shown).

The heparin-insensitive receptor for SF and HGF thus copurifies with the Met protein.

The β subunit of p190^{MET} receptor is cross-linked to the radiolabelled ligand

GTL16 cell monolayers were incubated with 1 nM [^{125}I]SF to equilibrium binding on ice, extensively washed, heparin eluted, treated with 1 mM of the homobifunctional cross-linking agent disuccinimidyl suberate (DSS) and extracted with detergent. The extract was then either analysed by SDS–PAGE and autoradiography as such or after immunoprecipitation with anti-Met-COOH and control non-

Table I. Invasion of cells into collagen gels (cells/cm^2)

	Factor added (10 ng/ml)		
Cell line	none	SF	HGF
A549	196[a]	3100	2900
HS766T	28	410	391
Capan 1	21	392	434

The invasion was measured by counting the number of cells[a] which entered the collagen matrix within 3 days by light microscopy. SF was as effective as HGF in promoting cell invasion.

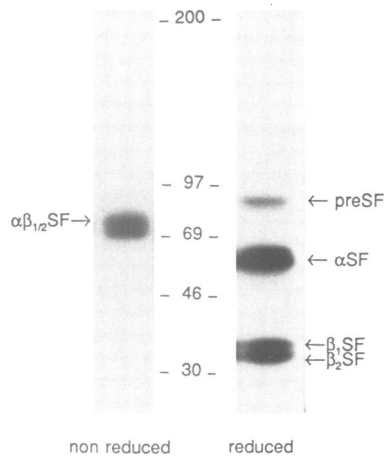

Fig. 3. Radiolabelled, pure SF analysed by SDS–PAGE and autoradiography. [^{125}I]SF was run under non-reducing and reducing conditions in a 5–15% gradient SDS–PAGE and exposed for autoradiography with intensifier screen for 2 h. SF migrates as a diffuse band with apparent M_r 74–78 kDa in non-reducing conditions. The preparation is virtually pure. Disulphide reduction resolves its three molecular components. The migration of ^{14}C-methylated molecular weight standards (in kDa) in reducing conditions is shown.

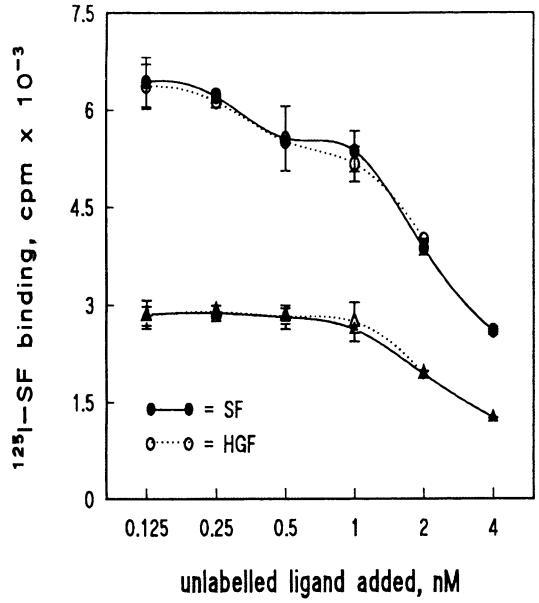

Fig. 4. SF and HGF bind to the same high and low affinity sites on A549 target cells. The low affinity binding site is eluted by heparin. A549 cell monolayers were incubated with 0.05 nM [^{125}I]SF in the presence of the indicated concentrations of unlabelled SF or HGF to equilibrium binding at 4°C. After extensive washing, the monolayers were extracted with 1% Triton X-100 and counted. The ligand displacement curves obtained with SF (closed circles, solid line) and HGF (open circles, broken line) are shown on the top half of the figure. On the bottom half of the figure, the response of the two binding sites to heparin elution is shown. After equilibrium binding as above, the monolayers were incubated for 5 min at 4°C with excess heparin. The eluate was collected and counted (closed triangles, solid line for SF; open triangles, broken line for HGF). Data shown are the means ± SEM of triplicate determinations from a representative experiment out of four performed.

immune serum (Figure 6). As shown in the figure, SF was cross-linked to a single major molecular species specifically recognized by the anti-Met-COOH antiserum (band 'a' in Figure 6). The whole $\alpha\beta$Met–$\alpha\beta$SF complex runs under non-reducing conditions faster than expected from the sum of the M_r of the single components. This reflects the behaviour of both SF and p190^{MET} when run individually, probably due to extensive intra- and inter-chain disulphide bonds (see Figure 3 and 5). After reduction of disulphide bridges, the cross-linked adduct was resolved in three distinct molecular species (labelled 'b','c', and 'd' in Figure 6) of M_r 240, 195 and 180 kDa, respectively. All three species contained the 145 kDa Met β subunit, as shown by their specific precipitation by the anti-Met-COOH. Their size could be explained by the cross-linking of the Met β subunit to the β subunit of SF for the smallest 'd' species (145 kDa + 32–36 kDa = 177–181 kDa), and to the α subunit of SF for the middle 'c' species (145 kDa + 55–65 kDa = 200–210 kDa). The larger 'b' adduct could be the complex of the cross-linked $\alpha\beta$ Met heterodimer and the SF β subunit (195 kDa + 32–36 kDa = 227–231 kDa) and/or the complex of the Met β subunit with the cross-linked $\alpha\beta$SF or the uncleaved preSF (145 kDa + 90 kDa = 235 kDa). Cross-linking of the uncleaved precursor is unlikely, as processing of the ligand molecule seems to be required for acquisition of the ability to bind the receptor (L.Naldini, unpublished observation). PreSF did not coprecipitate with the Met receptor and the 90 kDa labelled band in Figure 6 was dependent on the addition of the cross-linker. The

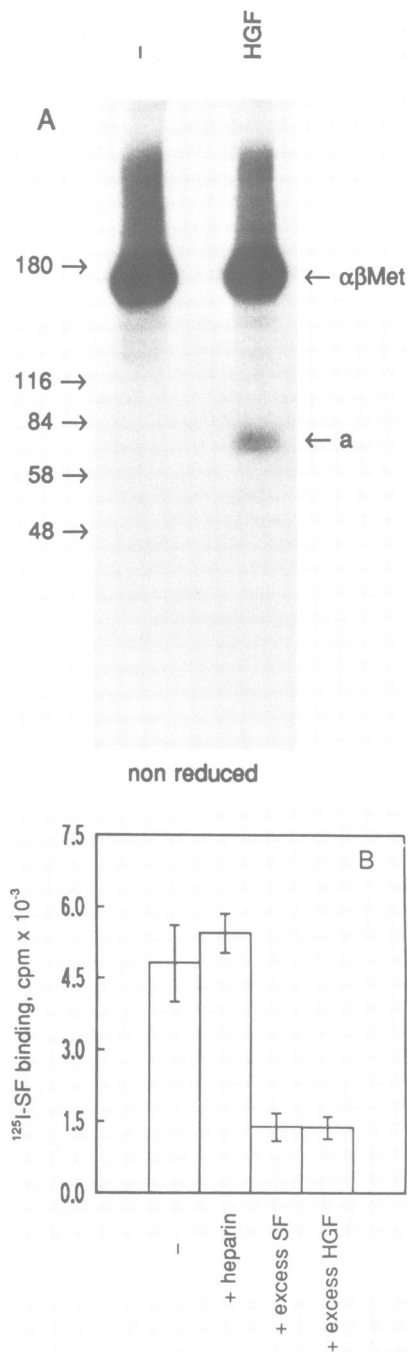

Fig. 5. SF and HGF bind to and coprecipitate with the Met receptor in immunocomplexes. The Met receptor was immunoprecipitated from GTL16 cell extract with anti-Met antibodies directed against the carboxy-terminus of the protein (anti-Met-COOH), as described in Materials and methods. In panel **A**, the cell monolayers were incubated without (–) or with unlabelled HGF to equilibrium binding at 4°C, extensively washed and surface-labelled with [^{125}I]iodine and lactoperoxidase-glucose oxidase, prior to extraction. The immunoprecipitates were analysed by 5–15% gradient SDS–PAGE and autoradiography with intensifier screen for 24 h. HGF coprecipitates with the Met protein, as shown by the band marked 'a' (apparent M_r 74–78 kd) in the figure. The migration of prestained molecular weight standards in kDa is indicated. In panel **B**, the specific binding of [^{125}I]SF to the immunoprecipitates of the Met protein is shown. 1 nM [^{125}I]SF was incubated with the immunocomplexes in the absence (–) or in the presence of the indicated competitors to equilibrium binding at 37°C. The total binding of [^{125}I]SF to 10 μl of Sepharose-bound immunocomplexes is shown. Data plotted are the means ± SEM of triplicate determinations from one of two similar experiments.

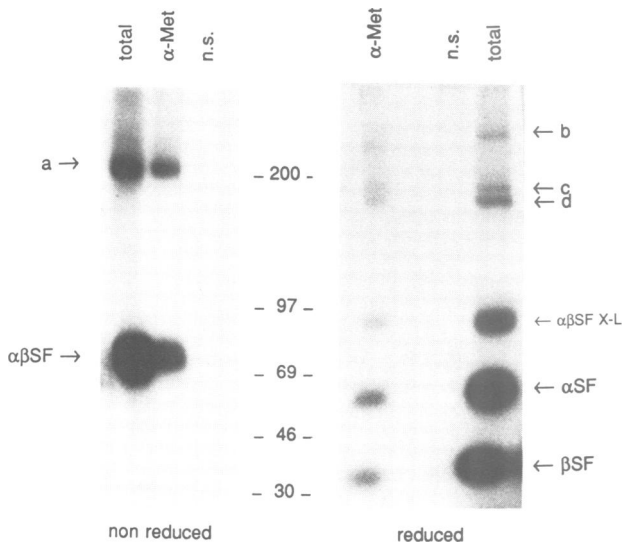

Fig. 6. Chemical cross-linking of [^{125}I]SF to the β subunit of the Met receptor. GTL16 cell monolayers were incubated with 1 nM [^{125}I]SF to equilibrium binding at 4°C, treated with 1 mM disuccinimidyl suberate (DSS) and extracted. The extract was then run as such (lanes marked 'total') or after immunoprecipitation with anti-Met-COOH antiserum and control non-immune serum (lanes marked 'α-Met' and 'n.s.', respectively) on 5–15% SDS–PAGE under non-reducing and reducing conditions and exposed for autoradiography with intensifier screen for 48 h. A single molecular species,'a', was labelled under non-reducing conditions and specifically recognized by the anti-Met-COOH antiserum. After reduction of disulphide bridges, it was resolved in three distinct molecular species, 'b' of 240 kDa, 'c' of 195 kDa, and 'd' of 180 kDa. All three species were precipitated by the anti-Met-COOH antiserum. The migration pattern of the labelled species in non-reducing conditions and their reduced M_r can be explained by the cross-linking of the 145 kDa Met β subunit to the three molecular components of SF. The migration of ^{14}C-methylated molecular weight standards (in kDa) under reducing conditions is shown.

uncleaved p170^{MET} precursor is not exposed at the cell surface in GTL16 cells (Giordano *et al.*, 1989b). Thus p170^{MET} could not contribute to any cross-linked adduct. Omitting the cross-linking agent or performing the reaction on cells not expressing the *MET* gene resulted in the disappearance of all labelled species except those representing the SF subunits (not shown).

When increasing concentrations of unlabelled SF and HGF were incubated together with 0.5 nM [^{125}I]SF prior to cross-linking, a dose-dependent decrease of the labelled cross-linked $\alpha\beta$Met–$\alpha\beta$SF complex was observed. HGF and SF were equally effective in reducing the intensity of the cross-linked band detectable by autoradiography (Figure 7).

These experiments show that the high affinity binding site for SF and HGF is located within or is closely associated with the β subunit of the Met receptor.

The receptor for SF and HGF is transferred to insect cells by a recombinant baculovirus carrying the MET cDNA

To obtain formal proof of the identity of p190^{MET} as receptor for SF and HGF, a recombinant baculovirus containing the human *MET* cDNA (Met–BCV) was constructed from the naturally occurring *Autographa californica* nuclear polyhedrosis virus. After infection with the recombinant Met–BCV, *Spodoptera frugiperda* insect cells (Sf9) expressed at their surface a fully processed Met protein, albeit with a slightly reduced M_r due to incomplete glycosylation (Ponzetto,C., Bardelli,A., Maina,F., Gout,I., Fry,M.J., Dhand,R., Waterfield,M. and Comoglio,P.M., in preparation).

As shown in Figure 8, Sf9 cells expressing the Met protein bound specifically [^{125}I]SF. Specific binding was not detected in control Sf9 cells infected with the wild-type virus. A ligand displacement curve was obtained incubating 0.1 nM [^{125}I]SF with increasing concentrations of unlabelled ligand and measuring the counts bound at equilibrium. Sf9 cells infected with the recombinant Met–BCV expressed a single class of binding site for [^{125}I]SF of nanomolar affinity.

Cross-linking experiments performed with 1 mM DSS after equilibrium binding of [^{125}I]SF identified the binding site with the expressed recombinant Met protein. As with the GTL16 cells (see Figure 6), a single high molecular weight complex was detected in Sf9 cells infected with the recombinant virus when the gel was run in non-reducing conditions ('a' in Figure 9). After reduction of disulphide bridges, the complex was resolved in three distinct molecular species, 'b' of M_r 230 kDa, 'c' of M_r 190 kDa, and 'd' of M_r 165–170 kDa. The M_r of the three labelled bands were consistent with the products of the cross-linking of the three components of SF to the β subunit of the recombinant Met protein, which has an M_r ~10 kDa lower than the native molecule.

Thus, expression of a recombinant Met protein is sufficient to reconstitute a high affinity receptor for SF in a completely heterologous system.

SF and HGF cDNAs have virtually identical sequences

A further appraisal of the identity between SF and HGF was carried out by molecular cloning of the respective cDNAs isolated from different sources.

Overlapping HGF cDNA clones were obtained from human liver mRNA by PCR. Oligonucleotides derived from the sequence of human liver HGF described by Nakamura *et al.* (1989) were used as primers for DNA amplification. Two partially overlapping clones were obtained, together encompassing the entire coding sequence (Figure 10B). Surprisingly, a number of nucleotide mismatches were found when the sequence was compared to that previously reported by Nakamura *et al.* (1989). This resulted in the replacement of 17 amino acid residues scattered throughout the coding region. Most of these amino acids substitutions (14 out of 17) were present as well in a HGF cDNA clone isolated from human placenta by Miyazawa *et al.* (1989). Furthermore, our sequence lacked a stretch of 15 nucleotides (481–495), causing an in-frame deletion of five amino acids. This deletion had already been described in HGF clones isolated from a human leukocyte cDNA library (Seki *et al.*, 1990) and obtained by PCR from a human embryonic lung fibroblast cell line (Rubin *et al.*, 1991).

SF cDNA clones were selected from a library prepared using mRNA from the human fibroblast MRC5 cell line. The library was screened with PCR products as probes. These were obtained using MRC5 cDNA as template and degenerated oligonucleotide primers. The oligonucleotides degenerate sequences were deduced from the amino acid sequence of two tryptic peptides derived from biologically active purified SF (Weidner *et al.*, 1990). A full-size and

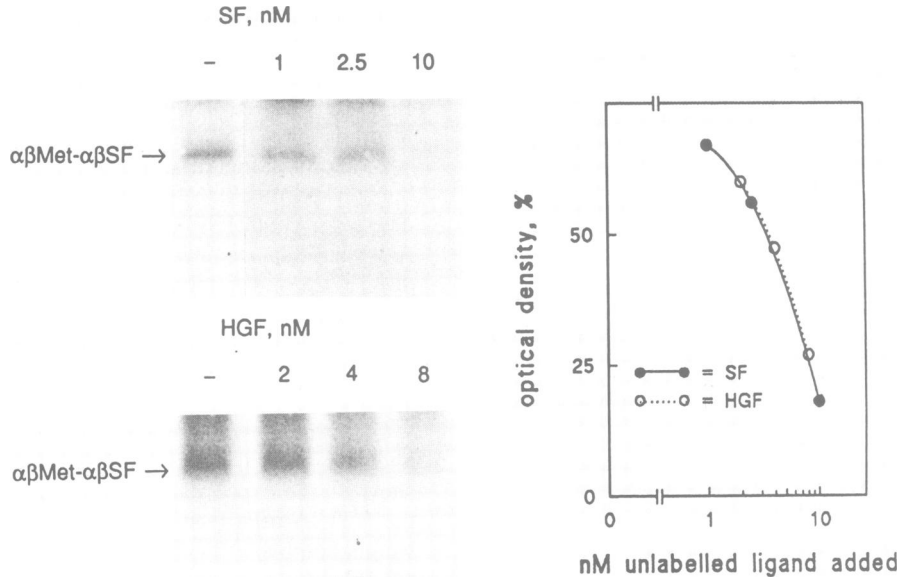

Fig. 7. SF and HGF compete with equal affinities for cross-linking to the Met receptor. GTL16 cell monolayers were incubated with the indicated concentrations of unlabelled SF or HGF together with 0.5 nM [^{125}I]SF to equilibrium binding at 4°C prior to cross-linking with 1 mM DSS as above. A dose-dependent inhibition of the labelling of the αβMet–αβSF complex detected by SDS–PAGE and autoradiography was observed. On the left, the relevant portions of the autoradiograms from a 24 h exposure with intensifier screen and pre-flashed films are shown. On the right, the absorbance of the cross-linked bands from the two autoradiograms is normalized relative to the control without competitor and plotted together against the concentration of unlabelled ligand.

a partial clone were isolated (Figure 10C and Weidner *et al.*, 1991). The full-size SF clone matched completely the sequence of the HGF clone isolated from a human placenta cDNA library by Miyazawa *et al.* (1989).

Using the full-size fibroblast SF clone as a probe, a number of different cDNA clones were also isolated from a library of human placenta. A full-size and three overlapping clones of different length were sequenced (Figure 10B). As in the case of fibroblast SF cDNA, the three partial clones from placenta showed complete identity with the HGF sequence reported by Miyazawa *et al.* (1989). The full-size clone showed the 15 bp deletion observed in the HGF clone that we isolated from liver by PCR.

Thus, the coding sequences of SF and HGF are indistinguishable. Two alternative transcripts differing for a stretch of five amino acids were detected in both cases.

The growth and motility responses to SF/HGF are mediated by the same receptor

We then asked whether the growth and motility responses elicited by SF/HGF in different target cells occurred through activation of the same receptor. Stimulation of the tyrosine phosphorylation of the β subunit of the Met protein by HGF was previously reported in cells responding to the factor with increased DNA synthesis (Bottaro *et al.*, 1991). SF and HGF were equally effective in stimulating tyrosine phosphorylation of the 145 kDa β subunit of the Met protein in GTL16 cells. No tyrosine phosphorylation was detected on the 170 kDa Met precursor (Figure 11).

A549 cells respond to SF/HGF with increased motility and matrix invasion (Figure 2 and Table I). However, their growth is not stimulated (data not shown). Immunoprecipitates of Met proteins prepared from A549 cells exposed to nanomolar concentrations of SF, and from control cells, were blotted and probed with phosphotyrosine antibodies. Stimulation with SF led to the phosphorylation on tyrosine of the 145 kDa β subunit of the Met receptor. The molecule comigrated with that stimulated in GTL16 cells (Figure 12).

Discussion

SF and HGF were originally identified as distinct molecules responsible for two different biological activities. However, their structure was found to be intriguingly similar. In this work we show that SF and HGF are indistinguishable ligands for the receptor encoded by the *MET* oncogene. The pure factors were interchangeable and equally effective in promoting hepatocyte growth, epithelial cell dissociation and matrix invasion. They bound with identical affinities to the same sites on target cells.

Analysis of the binding properties of SF and HGF indicated the existence of two classes of binding sites with affinities one order of magnitude apart. The lower affinity/large capacity site (K_d in the 10^{-9} M range) most likely corresponded to matrix- or cell-associated heparansulphate proteoglycans. This was indicated by: (i) the known ability of SF and HGF to bind heparin; (ii) the elution of the site by excess free heparin; (iii) the absence of heparin-sensitive binding site in the immunopurified Met protein. The existence of two classes of binding sites for HGF was previously reported (Zarnegar *et al.*, 1991) for rat hepatocytes, the lower affinity class being identified with heparin-like molecules. The affinity for heparin-like molecules can recruit SF/HGF to extracellular matrices or cell membranes. Among the possible functional consequences are ligand stabilization, induced fit for receptor binding, or conversely, block of biological activity due to ligand sequestering (Ruoslahti and Yamaguchi, 1991).

The higher affinity binding site for SF and HGF (K_d in the 10^{-10} M range) was identified as the receptor encoded

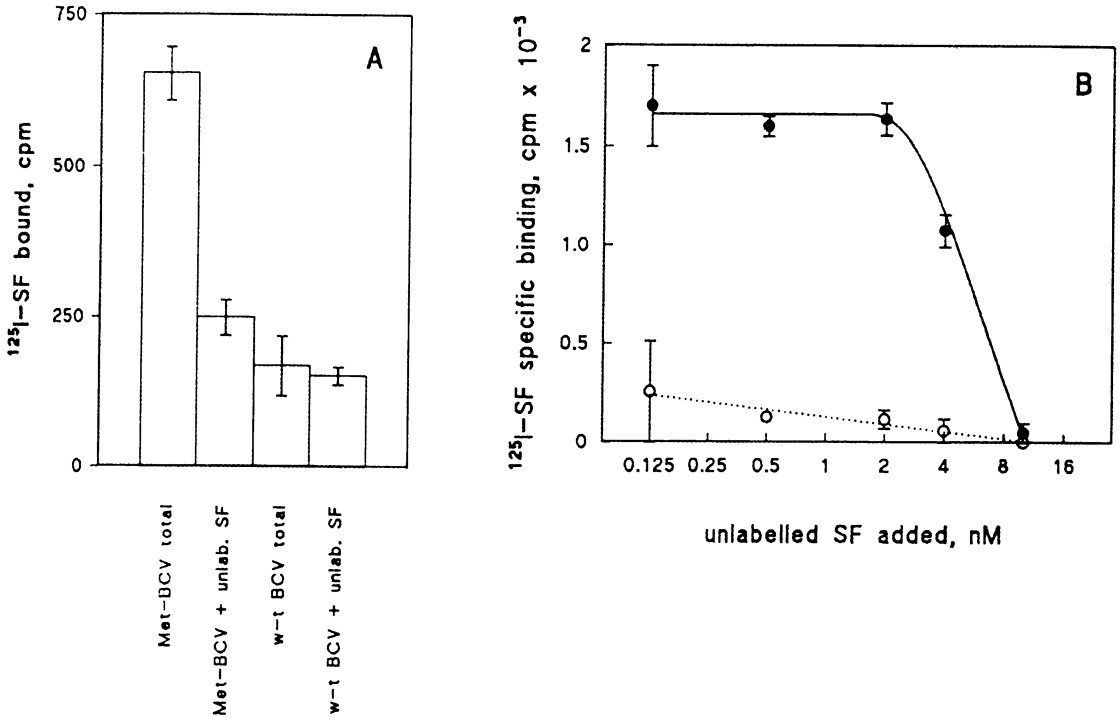

Fig. 8. The binding activity for SF/HGF is transferred to insect Sf9 cells by infection with a recombinant baculovirus carrying the *MET* cDNA. A recombinant baculovirus containing the human *MET* cDNA (Met−BCV) was constructed from the wild-type nuclear polyhedrosis virus (w-t BCV). After infection with the recombinant Met−BCV, insect Sf9 cells acquire the capacity to bind human [^{125}I]SF with high affinity. Panel **A** shows the total binding of 0.1 nM [^{125}I]SF to Sf9 cells infected with either virus, after reaching equilibrium at 4°C in the absence or in the presence of a 100-fold excess of unlabelled SF. Each point represents the binding to 5×10^5 cells. Panel **B** shows the ligand displacement curve obtained by incubating Sf9 cells infected with the recombinant Met−BCV (closed circles, solid line) or the wild-type virus (open circles, broken line) with 0.1 nM [^{125}I]SF and the indicated concentrations of unlabelled ligand and measuring the counts bound at equilibrium. Specific binding was calculated by subtracting from the total the counts bound in the presence of a 100-fold excess (10 nM) of unlabelled ligand. Each point represents the specific binding to ~10^6 cells. Data plotted in both panels are the mean and variation range of replicate determinations of two similar experiments.

by the *MET* oncogene. This had been previously suggested by the ability of HGF to stimulate tyrosine phosphorylation of p190^{MET} in intact cells and *in vitro* using the partially purified protein (Bottaro *et al.*, 1991; Naldini *et al.*, 1991a). Cross-linking to the Met protein β subunit of a molecule smaller than HGF (M_r 28 kDa) but with similar binding properties had also been reported (Bottaro *et al.*, 1991). Using pure radiolabelled SF we now show: (i) heparin-insensitive, specific binding to immunopurified p190^{MET}; (ii) chemical cross-linking of the α and β chains of the ligand to the β subunit of the Met receptor; (iii) equal ability of unlabelled SF and HGF to compete for binding and cross-linking the Met receptor. SF and HGF were also equally effective in stimulating the tyrosine phosphorylation of the Met β subunit in GTL16 cells. This result represents the functional counterpart to the cross-linking experiments in proving the interaction of SF and HGF with the *MET* receptor.

Formal proof of the role of p190^{MET} as the sole component of the SF/HGF receptor was obtained by reconstituting the ligand binding site in insect cells infected by a recombinant baculovirus carrying the *MET* cDNA. A single class of binding site was observed with nanomolar affinity. This was a slightly lower value than that calculated for the native receptor. A similar decrease in affinity has also been reported for recombinant EGF receptor (Greenfield *et al.*, 1988) and for *trk*/NGF receptor (Klein *et al.*, 1991) when expressed in insect cells. This could be due to differences in the binding site caused by the incomplete glycosylation occurring in this expression system. Other explanations could involve the different membrane fluidity or composition of insect cells.

The identity of SF and HGF was proved by molecular cloning of their cDNAs from fibroblasts, placenta and liver cells. The occurrence of two alternative transcripts, one of which carries a 15 bp in-frame deletion, was observed both for SF and HGF. Whether these alternative transcripts generate two proteins with distinct biological activity is at present unknown. However, we could not detect any difference in the biological activity of SF or HGF purified from different sources. Evidence for the identity between HGF and SF including the existence of a gene present in a single copy has also been reported by some of us (Weidner *et al.*, 1991).

A common transducing mechanism was shown to operate at the receptor level in cells responding to HGF and SF with growth versus increased cell motility and matrix invasion. Tyrosine phosphorylation of the Met receptor β subunit was stimulated in both cases. This event marks activation of the receptor (Ullrich and Schlessinger, 1990; Naldini *et al.*, 1991b). Different biological responses must therefore result from divergence within the cascade of biochemical events occurring downstream from the receptor in different target cells.

The occurrence of two distinct transcripts of the *MET* oncogene due to alternative splicing in the region corresponding to the extracellular domain has been described (Ponzetto *et al.*, 1991; Rodriguez *et al.*, 1991). This prompted us to consider the possible existence of two Met receptor isoforms which could mediate the different

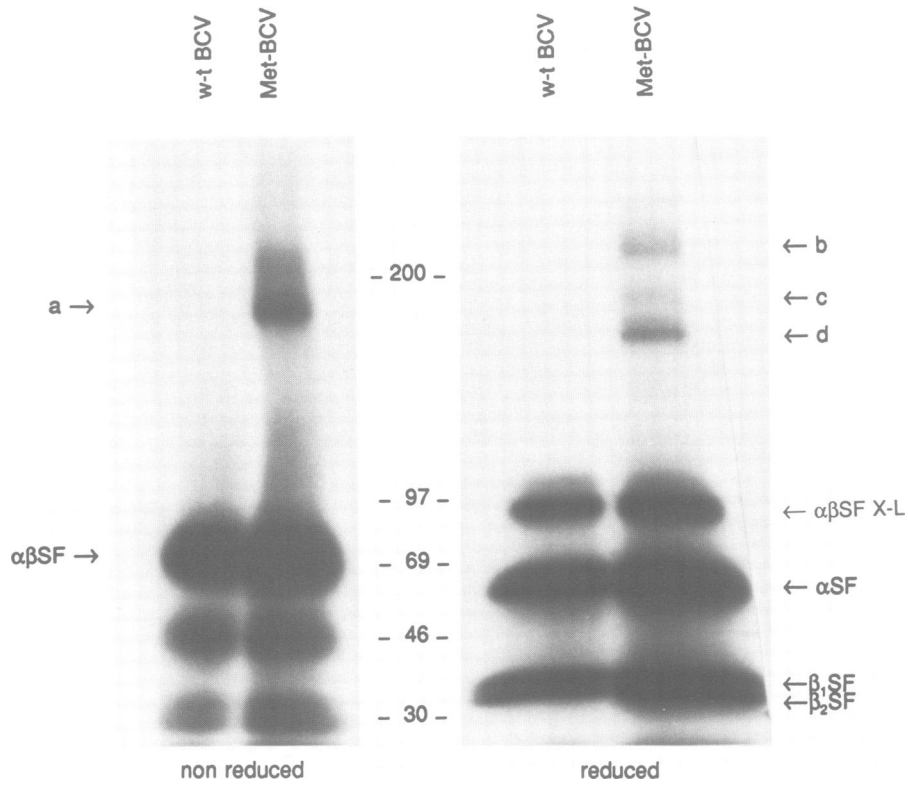

Fig. 9. Chemical cross-linking of [^{125}I]SF to the Met receptor expressed in Sf9 insect cells. Sf9 cells infected either with the wild-type (w-t BCV) or the recombinant baculovirus carrying the *MET* cDNA (Met−BCV) were incubated with 1 nM [^{125}I]SF to equilibrium binding at 4°C, and treated with 1 mM disuccinimidyl suberate. The cell extract was run on 5−15% gradient SDS−PAGE under non-reducing and reducing conditions and exposed for autoradiography with intensifier screen for 5 days. As with GTL16 cells (see above Figure 6), a single high molecular weight labelled band was evident in Sf9 cells infected with Met−BCV under non-reducing conditions ('a'). After reduction of disulphide bridges, it was resolved in three distinct molecular species, 'b' of M_r 230 kDa, 'c' of M_r 190 kDa, and 'd' of M_r 165−170 kd. The migration pattern of the labelled species in non-reducing conditions and their reduced M_r can be explained by the cross-linking of the recombinant Met β subunit, which has a M_r ~10 kDa lower than the native molecule, to the three molecular components of SF. The migration of ^{14}C-methylated molecular weight standards under reducing conditions is shown.

biological responses. The original cDNA clone of the human *MET* gene (Park *et al.*, 1987) represents a rare transcript which, in a transient expression system, yields an unprocessed receptor of M_r 170 kDa (Rodriguez *et al.*, 1991). In the cell lines tested by us so far, however, all the detectable p170^{MET} appears to be the biosynthetic precursor of the mature Met heterodimer (Giordano *et al.*, 1989b). Accordingly, p170^{MET} is not exposed at the cell surface, nor is it phosphorylated on tyrosine upon ligand stimulation. We cannot formally rule out the possibility that a very limited amount of a Met isoform of M_r 170 could actually escape detection and be present on the plasma membrane. The binding and cross-linking experiments, however, indicate only one high affinity binding site and only one major labelled species in the target cells tested. These include insect cells infected with Met−BCV, where an unprocessed precursor accumulates, and is in part exposed at the cell surface, together with the dimeric Met receptor (C.Ponzetto, in preparation).

On the other hand, a Met protein truncated immediately distal to the transmembrane domain and a soluble, secreted molecule structurally and immunologically indistinguishable from the extracellular portion of the p190^{MET}, were recently described (Prat,M., Crepaldi,T. and Comoglio,P.M., submitted for publication). These Met forms originate in different cell lines from proteolysis of the mature p190^{MET}. While they cannot contribute to the intracellular transduction of the SF/HGF signal, as they are devoid of the kinase domain, they could interfere with this signalling pathway, by subtracting the ligand from the functional Met receptor. Alternatively, they could participate in ligand transport and contribute to the low affinity cell surface binding of SF and HGF discussed above.

The *MET* oncogene is widely expressed in normal epithelial tissues (Iyer *et al.*, 1990; Naldini *et al.*, 1991a; Prat *et al.*, 1991). Its level of expression is often increased with neoplastic transformation (Di Renzo *et al.*, 1991). Amplification of the *MET* oncogene has been reported both in cell lines derived from human tumours (Giordano *et al.*, 1989a) and as a common event in the adaptation of cells to *in vitro* culture (Cooper *et al.*, 1986). HGF expression has been detected in many tissues (Zarnegar *et al.*, 1990; Tashiro *et al.*, 1990), often with a parallel distribution to that of the *MET* gene. The recognition of the identity of HGF with SF, in view of the well-documented stromal origin of SF, opens the way to the dissection of novel paracrine and possibly autocrine circuits involved in the control of normal and neoplastic cell growth, motility and invasion.

Materials and methods

Reagents, antibodies and cell lines

All reagents used were of analytical grade. Protease inhibitors and bovine serum albumin (BSA), crystalline, cell culture tested, were purchased from Sigma. *S.aureus* Protein A covalently coupled to Sepharose was purchased

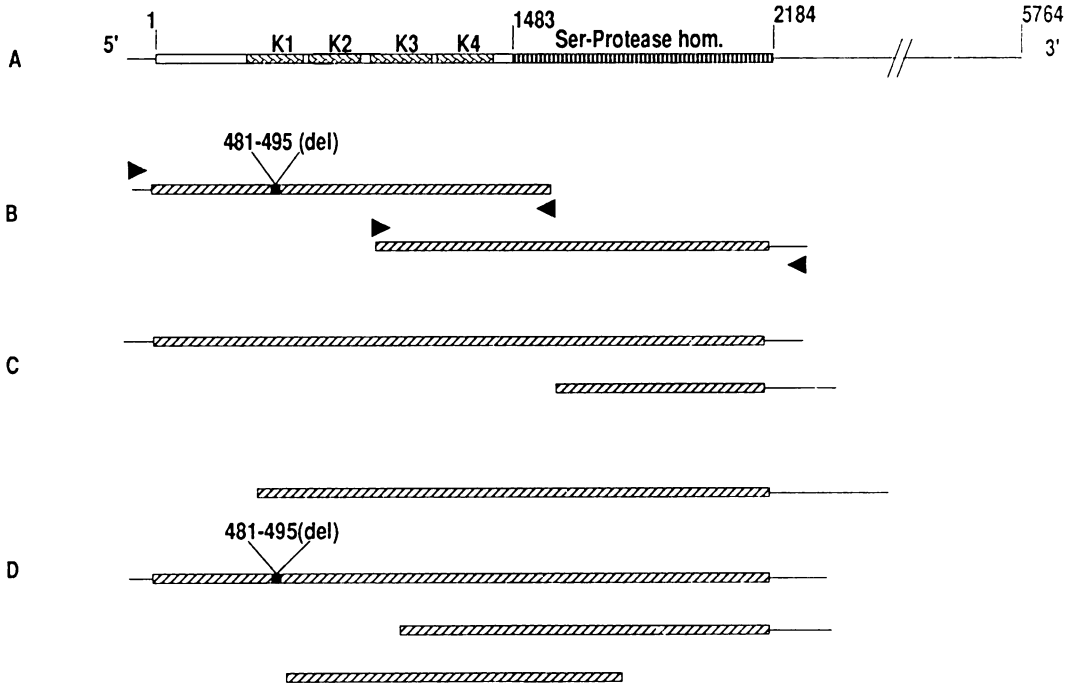

Fig. 10. Schematic representation of the HGF and SF clones obtained from human liver, MRC5 fibroblasts and placenta. **A**. HGF cDNA according to Nakamura *et al.* (1989). The coding region is boxed, kringles and protease domains are indicated. **B**. Overlapping PCR clones from human liver mRNA. The 15 nucleotide deletion between positions 481 and 495 in the first kringle region is outlined. The oligonucleotide primers used for the amplification are indicated by arrowheads. **C**. Full size and partial SF clones from MRC5 cDNA library. **D**. Overlapping clones isolated from the human placenta cDNA library. The full size clone shows the same deletion found in the PCR HGF clone from human liver.

from Pharmacia. Reagents for SDS−PAGE and nitrocellulose filters were from Bio-Rad. Carrier free [^{125}I]Na, [^{125}I]protein A, [6-^{3}H]thymidine, and ^{14}C-methylated molecular weight standards used in SDS−PAGE—myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (69 kDa), egg albumin (46 kDa) and carbonic anhydrase (30 kDa)—were obtained from Amersham. Prestained molecular weight standards were from Sigma.

Phosphotyrosine antibodies were raised against *p*-amino-benzene-phosphonate and affinity-purified as previously described (Comoglio *et al.*, 1984). Anti-Met antibodies were raised in rabbits, or were purified from ascitic fluids of hybridoma from mice, immunized against the synthetic peptide VDTRPASFWETS corresponding to the amino acid sequence at the C-terminal end of the predicted *MET* gene product and kindly provided by M.F.Di Renzo, T.Crepaldi and M.Prat.

GTL16 cell line is a clonal cell line derived from a poorly differentiated gastric carcinoma line (Motoyama *et al.*, 1984). A549 lung carcinoma cells was obtained from ATCC. HS766T and Capan 1 pancreas carcinoma cell lines were from the tumour cell bank of the German Cancer Research Center (DKFZ), Heidelberg. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Assays for cell growth, motility and matrix invasion

Primary cultures of rat hepatocytes were prepared as described (Zarnegar *et al.*, 1991) and seeded on collagen (Boehringer)-coated 6-well plates in M199 medium supplemented with 100 ng/ml insulin. Medium was changed after 4 h and the cultures used within 48 h. The factors were added at the indicated concentrations for 24 h, then [6-^{3}H]thymidine (28 Ci/mmol, 1 μCi/ml) was added in fresh medium and the cultures further incubated for 18 h. Cell growth was evaluated by [^{3}H]thymidine incorporation into TCA-precipitable material.

The dissociation assay on MDCK cells and A549 cells was performed as previously described (Stoker *et al.*, 1987; Weidner *et al.*, 1990). Cells were seeded at low density in 6-well plates and exposed to the indicated concentrations of factors in fresh medium overnight. The scattering effect was monitored by light microscopy. 1 U of activity was defined as the lowest amount of factor per ml that clearly dissociated MDCK cells.

Invasion of collagen matrices was performed according to Behrens *et al.* (1989). Cells which entered the collagen matrix were counted after 3 days by light microscopy. New factor was added daily in fresh medium.

Ligand radioiodination and binding assays

Pure SF (1 μg) was radiolabelled with carrier-free [^{125}I]Na (2 mCi) and Iodogen (Pierce). 200 μl of Iodogen at 100 μg/ml in chloroform were dried in a polypropylene vial under nitrogen flow. SF and ^{125}I were then added in 0.25 M phosphate buffer pH 7.4. The reaction was allowed to proceed for 15 min at 4°C, then the mixture was transferred to another vial and left on ice for 10 min. Carrier BSA was added to a final concentration of 0.1% in 0.4 M NaCl, 0.1% CHAPS, 20 mM PO_4 buffered to pH 7.4 and the labelled ligand was fractionated from the free [^{125}I]Na by affinity chromatography on a 1 ml heparin−Sepharose column (Pierce) pre-equilibrated with the same buffer. After extensive washing,the column was eluted with 1.3 M NaCl in the same buffer and 0.5 ml fractions were collected. Fractions containing TCA-precipitable radioactivity were pooled, concentrated with a Centrisart (Sartorius) diafiltration apparatus with a membrane cut off of 20 kDa and stored at 4°C. The specific activity of the tracer was $\sim 8\times10^{7}$ c.p.m./μg (5700 Ci/mmol), corresponding to a I:SF molar ratio of around 3:1. Thus, the preparation did not contain a significant amount of unlabelled molecules.

For binding studies, cells were seeded at low density on collagen-coated microwells and shifted to medium without serum after 24 h. After 24 h of serum starvation, the monolayers were put on ice, rinsed four times with prechilled RPMI medium containing 20 mM HEPES pH 7.4, 0.1% BSA, 100 μg/ml soybean trypsin inhibitor and bacitracin (binding medium), and incubated with 0.05 nM [^{125}I]SF in binding medium, without or with the indicated concentrations of competitors, for 3 h at 4°C in a final volume of 200 μl/well. The monolayers were then washed five times with the binding medium and either directly extracted with 1% Triton X-100 in PBS or pre-eluted with 0.1 mg/ml heparin in binding medium for 5 min at 4°C and then extracted with Triton X-100. Both the heparin-eluate and the Triton-extract were then counted in a Packard γ-counter. Total binding was < 10% of the added c.p.m. and specific binding (calculated by subtracting from the total the c.p.m. bound after incubation with a 100-fold excess of unlabelled ligand) was ~60%. Approximate estimates of receptor K_d could be obtained by ligand displacement curves, as the concentration of ^{125}I-labelled ligand was more than 4-fold smaller than the estimated K_d and the tracer preparation did not contain a significant amount of unlabelled molecules. However, because of the slightly reduced biological activity of [^{125}I]SF, the binding capacities could not be reliably estimated.

Immunoprecipitation and binding to immunocomplexes

Cell monolayers were grown in 78.5 cm^2 dishes and incubated with unlabelled ligand to equilibrium binding at 4°C as described above. After extensive washing and heparin elution, the monolayers were surface-iodinated with 1 mCi/dish [^{125}I]Na and the lactoperoxidase, glucose oxidase method, in PBS at 4°C. After 20 min, the radiolabelling was stopped by washing

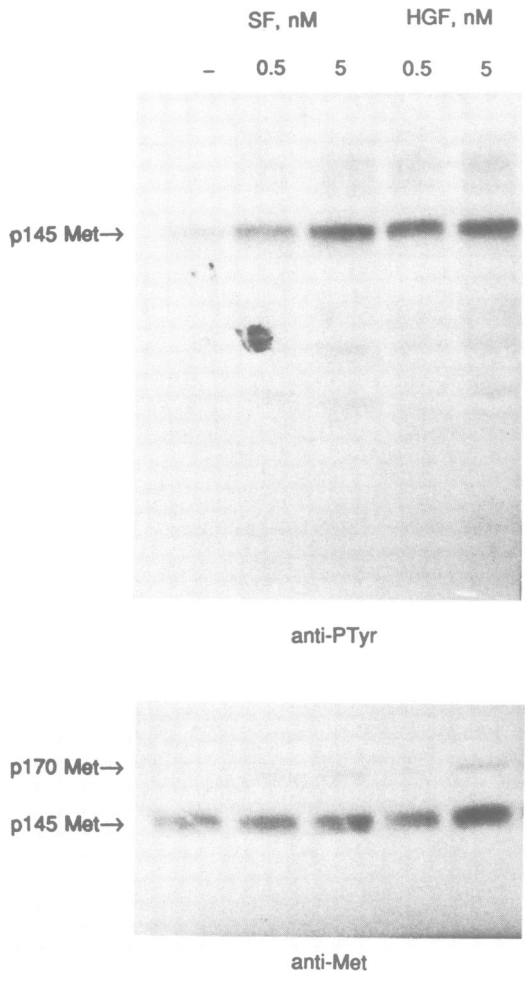

Fig. 11. SF and HGF are equally effective in stimulating the phosphorylation on tyrosine of the 145 kDa Met β subunit in GTL16 cells. Serum-starved GTL16 cells were incubated for 10 min at 37°C without (−) or with the indicated concentration of SF or HGF. The monolayers were then solubilized in boiling Laemmli buffer and protein-matched equal 100 μg aliquots from each sample were run in 8% SDS−PAGE under reducing conditions, transferred onto nitrocellulose and probed with 10 μg/ml either of purified anti-phosphotyrosine antibodies (top panel, anti-PTyr) or anti-Met-COOH antibodies (bottom panel, anti-Met), followed by ^{125}I-labelled protein A and autoradiography overnight at −70°C with intensifier screen. The 145 kDa Met β subunit (p145 Met) and its 170 kDa precursor (p170 Met) are labelled by the anti-Met antibodies but only the 145 kDa subunit of the mature Met heterodimer is detectably phosphorylated on tyrosine upon ligand stimulation. Its basal level of tyrosine phosphorylation is the consequence of the amplification and overexpression of the *MET* gene in GTL16 cells.

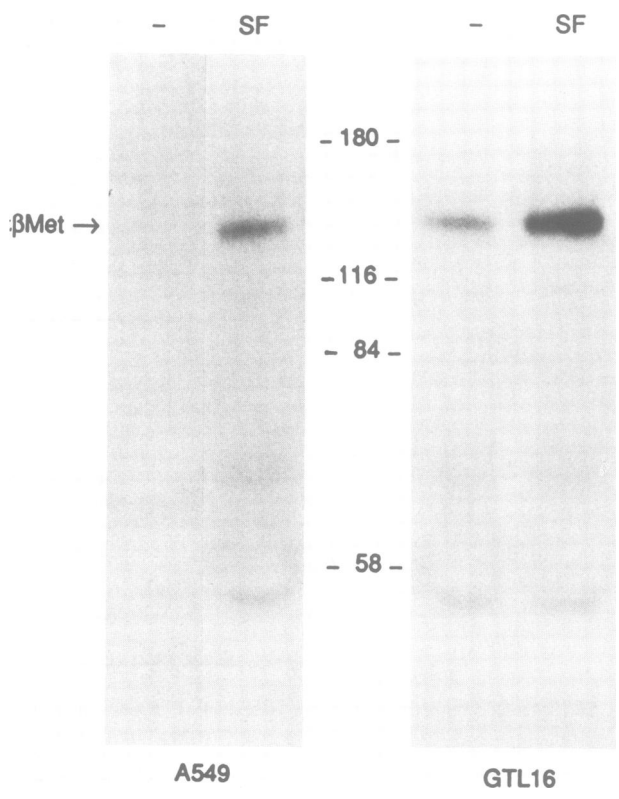

Fig. 12. SF stimulates the phosphorylation on tyrosine of the 145 kDa β subunit of the mature Met heterodimer in different target cells. Serum-starved A549 and GTL16 cell monolayers were preincubated with 35 μM phenylarsine oxide for 10 minutes, incubated without (−) or with 2.5 nM SF (SF) for 10 min at 37°C and extracted with boiling lysis buffer containing 1% SDS, EDTA and a cocktail of protease and phosphatase inhibitors. After dilution in a CHAPS-containing buffer, the lysates were immunoprecipitated with anti-Met-COOH antibodies and blotted with anti-phosphotyrosine antibodies as above. The blots were exposed for autoradiography overnight at −70°C with intensifier screen. The phosphorylation on tyrosine of the 145 kDa Met β subunit (βMet) is stimulated in both cell types. As in Figure 11 above, a basal level of phosphorylation is observed in GTL16 cells. The migration of prestained molecular weight standards (in kDa) is shown.

the monolayers twice with PBS containing 0.02% NaN_3 and the cells were scraped off with a rubber policeman into 0.4 ml of ice-cold HEPS buffer (1.25% CHAPS in 25 mM HEPES−NaOH pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 10% glycerol v/v, 10 mM iodoacetamide and a cocktail of protease inhibitors including 1 mM PMSF, 50 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 10 μg/ml pepstatin). The extraction was continued for 20 min at 4°C with stirring. The cell lysates were cleared by centrifugation at 15 000 *g* for 20 min at 4°C, and immunoprecipitated with Sepharose−protein A precharged with either anti-Met antiserum or control serum for 1 h at 4°C with stirring. Bound proteins were washed five times with HEPS buffer, eluted with boiling Laemmli buffer (Laemmli, 1970) without reducing agent, run in a 5−15% gradient SDS−PAGE and exposed for autoradiography at −70°C using intensifying screens.

For binding experiments, immunoprecipitates were prepared as above from untreated cells with purified monoclonal antibodies directed against a carboxy-terminal peptide of the Met protein (DR6) covalently linked at a high coupling ratio to Sepharose−protein A with 20 mM dimethylpimelimidate. A small volume of beads (10 μl/0.2 ml cell extract) were then incubated with GTL16 cell extract, extensively washed and further incubated with 1 nM [^{125}I]SF for 25 min at 37°C in the absence or presence of different competitors in binding medium. The beads were then washed extensively with binding medium and counted in a γ-counter.

Chemical cross-linking

Cell monolayers were grown and incubated with 1 nM ^{125}I-labelled ligand to equilibrium binding at 4°C as described above. After extensive washing and heparin elution, the monolayers were treated with 1 mM disuccinimidylsuberate (DSS, Pierce) for 20 min at 4°C. The reaction was stopped by addition of Tris to a final concentration of 20 mM, the dishes were washed twice with PBS and extracted with HEPS buffer as above. The cleared lysate was either immunoprecipitated with anti-Met antibodies as above and/or directly run in 5−15% gradient SDS−PAGE after boiling in Laemmli buffer. Gels were exposed for autoradiography at −70°C and the intensity of the cross-linked bands was estimated by densitometric scanning of the film with a LKB 2202 Ultroscan laser densitometer.

Expression of MET cDNA in insect cells using a baculovirus vector

Spodoptera frugiperda (Sf9) cells, obtained from the American Type Culture Collection, were grown in monolayer cultures using IPL-41 medium (Gibco). The full-length *MET* cDNA was cloned into the baculovirus pAcC4 vector, a derivative of pAcC3 (Max D.Summers, Texas A&M University). Using the Lipofectin (BRL) technique, the recombinant vector was cotransfected

with wild-type baculovirus DNA (AcNPV, Max D.Summers) into Sf9 cells. The resulting recombinant virus was used to infect fresh Sf9 cells. Seven days later, positive clones were identified by dot blot. Details of the purification procedure and full characterization of the recombinant protein will be described elsewhere (C.Ponzetto *et al.*, manuscript in preparation). The recombinant protein was identified through immunoprecipitation with specific antibodies, *in vitro* kinase assay, Western blotting with anti-Met and anti-phosphotyrosine antibodies, and surface labelling of Sf9 cells with ^{125}I. A fully processed Met protein is present on the Sf9 cell surface, together with a fraction of the uncleaved precursor, which accumulates at late times after infection. For binding experiments with [^{125}I]SF, Sf9 cells were used 48 h after infection with recombinant or wild-type virus at high multiplicity.

Western blotting

Serum-starved cell monolayers were incubated for 10 min at 37°C with prewarmed binding medium in the absence or in the presence of the indicated concentrations of ligand. Tyrosine phosphorylation of total cellular proteins was analysed by Western blotting of whole cell lysates in boiling Laemmli buffer with anti-phosphotyrosine antibodies as previously described (Di Renzo *et al.*, 1986). For analysis of immunoprecipitates, the monolayers were incubated with 35 μM phenylarsine oxide (Fluka) for 10 min at 37°C (Bernier *et al.*, 1987; Garcia-Morales *et al.*, 1990) prior to stimulation. The cells were then lysed in boiling HEDS buffer (50 mM HEPES pH 7.6, EDTA 10 mM, glycerol 10%, CHAPS 1%, Na pyrophosphate 50 mM, NaF 100 mM, Na orthovanadate 2 mM, phenylphosphate 30 mM, $ZnCl_2$ 1 mM, ammonium molybdate 50 μM and the protease inhibitors above cited) supplemented with 1% SDS. After a brief sonication, the lysates were diluted 10-fold with HEDS buffer, cleared by ultracentrifugation and immunoprecipitated with anti-Met antibodies, eluted with Laemmli buffer, subjected to SDS–PAGE and transferred onto nitrocellulose paper by high-intensity wet blotting. Blots were probed with 10 μg/ml of purified anti-phosphotyrosine or anti-Met antiserum followed by ^{125}I-labelled protein A. Filters were subjected to autoradiography overnight at −70°C using intensifying screens.

Molecular cloning of HGF and SF cDNA

Amplification by PCR of liver HGF sequences was performed on single-stranded DNA templates obtained from ~10 μg of human normal liver mRNA by extending the appropriate 3′ primer (50 pmol) with M-MLV reverse transcriptase (BRL) at 37°C for 30 min. Heat inactivation of the enzyme was followed by addition of Taq or Vent polymerase and the appropriate 5′ primer (35 pmol) to an aliquot of the reverse transcription product. The concentration of the previously added 3′ primer was equalized to that of the 5′ primer. The reaction mix for Taq polymerase (Promega) amplification consisted of 10 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5–6.0 mM $MgCl_2$ and the four dNTPs at 0.2 mM. For the amplification with Vent polymerase (Biolabs), endowed with proof-reading activity, the buffer provided by the manufacturer was used. Thirty PCR cycles were performed in a Programmable Thermal Controller (MJ Research) with a step cycle profile of 1 min at 92°C, 2 min at 50°C and 2 min at 72°C. The PCR products were then purified in low melting agarose and cloned in Bluescript plasmid vector (Stratagene). DNA sequencing was performed on double-stranded plasmids using the deoxynucleotide procedures described in the Sequenase 2.0 (United States Biochemical) or in the T7 Sequencing (Pharmacia) kits. Specific oligonucleotide and Bluescript Universal and Reverse primers were used to sequence both strands of two individual clones containing the HGF coding sequence. Specific oligonucleotide primers were synthesized by standard phosphoramidite methods with a PCR-Mate 391 DNA Synthesizer (Applied Biosystem).

A PCR product was generated from human MRC5 fibroblast cDNA using degenerated oligonucleotide primers designed from SF tryptic peptides (Weidner *et al.*, 1991). The step cycle profile of this amplification with Taq polymerase was of 2 min at 94°C, 3 min at 55°C and 5 min at 72°C. This PCR fragment was used to isolate SF cDNA clones from a human MRC5 fibroblast cDNA library constructed in λgt10 (Birchmeier *et al.*, 1990). A full-size SF clone from human MRC5 fibroblast library was used to isolate several clones from a cDNA library obtained from placenta.

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