Hepatocyte Growth Factor/Scatter Factor Has Distinct Classes of Binding Site in Heparan Sulfate from Mammary Cells†

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ABSTRACT: Hepatocyte growth factor/scatter factor (HGF/SF) is a heparan sulfate (HS)-binding growth factor and morphogen for mammary epithelial cells that is produced by mammary stromal fibroblasts. HS chains, purified as peptidoglycans from a panel of cell lines representative of the ductal epithelial cell (Huma 123), the myoepithelial cell (Huma 109), the stromal fibroblast (Rama 27), and malignant mammary epithelial cells (MCF-7 and ZR-75), were used in a biosensor-based assay to identify the classes of HGF/SF-binding sites in the polysaccharide chains. At least three distinct binding sites were identified. One site exhibits fast association and fast dissociation kinetics [k_{ass} (1.4−7.7) × 10^6 M^{-1} s^{-1}; k_{diss} 0.0032−0.0096 s^{-1}] and is present on the HS from benign Huma 123 epithelial cells, Huma 109 myoepithelial-like cells, and ZR-75 malignant cells. The second binding site, found on HS from the malignant MCF-7 cells, has slower HGF/SF-binding kinetics (k_{ass} 0.20 × 10^6 M^{-1} s^{-1}; k_{diss} 0.00055 s^{-1}). The third binding site possesses fast association and slow dissociation kinetics (k_{ass} 1.1 × 10^6 M^{-1} s^{-1}; k_{diss} 0.00020 s^{-1}) and was found on the HS isolated from the culture medium of the Huma 123 benign epithelial cells. The first and second binding sites have a similar K_d, 1−3 nM, while the third binding site has a considerably higher affinity for HGF/SF (K_d 200 pM). The three binding sites seem to be mutually exclusive, since each sample of HS possessed just one of the sites.

The glycosaminoglycan heparan sulfate (HS)[1] is present on the surface and extracellular matrix of most metazoan cells and is usually linked to a core protein to form HS proteoglycans (HSPGs) (*1*, *2*). The HS chains are unbranched and consist of repeating disaccharide units of glucosamine and glucuronic acid or its epimer iduronic acid, substituted to varying extents with aminoacetyl and ester and amino sulfate groups. Since over 10 structurally distinct monosaccharides have been characterized, the sequence complexity of HS is akin to that of proteins at the amino acid level. A considerable number of growth factors have been found to bind specifically to the HS chains of HSPGs (*1*−*5*).

The growth factor-binding activity of HS can have several functions. The sequestration of a growth factor on extracellular HS will prevent its diffusion and allow a local store to accumulate and act on a restricted number of cells (*6*). In addition, certain growth factors, notably the fibroblast growth factors and hepatocyte growth factor/scatter factor (HGF/SF), are dependent on HS for their activity. These growth factors require both their cognate tyrosine kinase receptors and their HS receptors to be present, if they are to stimulate cell division or cell migration (*7*−*13*).

The development of the mammary gland has been widely studied, and both growth and patterning persist from birth through to puberty (*14*, *15*). This results in the formation of the mature virgin mammary gland, which consists of a ductal tree, terminating in alveolar buds: the lumen is lined by epithelial cells with myoepithelial cells on the basolateral surface of the gland, abutting the basement membrane. Components of the basement membrane are largely deposited by the myoepithelial cells and stromal fibroblasts. The gland subsequently undergoes cycles of development and regression in the adult, associated with pregnancy/lactation and weaning/involution, respectively [reviewed in (*14*, *15*)]. HGF/SF is a stroma-derived growth factor, which is implicated in regulating the development of the gland (*16*). Moreover, in collagen gels, mammary epithelial cells can be induced to form tube-like structures (*17*), reminiscent of the ducts seen in vivo; HGF/SF is a potent stimulator of the formation of such structures in this system (*18*, *19*).

In view of the dependence of the activity of HGF/SF on HS, it seems likely that the HS species produced by mammary cells will play a key developmental role. Moreover, since HGF/SF is a paracrine effector, produced in the mammary stroma and acting on the epithelium, HS receptors could potentially regulate the delivery of the growth factor to the target cells. We have used a biosensor-based binding

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[1] Abbreviations: bFGF, basic fibroblast growth factor; HS, heparan sulfate; HSPG, HS proteoglycan; HGF/SF, hepatocyte growth factor/scatter factor; Huma, human mammary; PBS, phosphate-buffered saline (137 mM NaCl, 10 mM Na_2HPO_4, pH 7.2); PBST, PBS with 0.02% Tween 20; Rama, rat mammary.

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assay (*20*, *21*) to measure quantitatively the binding parameters of HGF/SF for HS produced by cells representative of the ductal epithelial cell, the myoepithelial cell, the stromal fibroblast, and malignant mammary tumors. The results indicate that the HS from the mammary cells possesses at least three distinct classes of binding sites for HGF/SF and imply that these receptors may serve to control the activity of HGF/SF in the mammary gland.

MATERIALS AND METHODS

Materials and Cells. Recombinant human HGF/SF was obtained from R&D Systems (Abingdon, U.K.). Chondroitinase ABC, Pronase, and micrococcal nucleases were obtained from Sigma (Poole, U.K.). Sialidase enzyme collection (EC 3.2.1.18) was from Oxford GlycoScience (Oxford, U.K.) and endo-β-galactosidase (EC 3.2.1.03) and bovine pancreatic RNase were from Boehringer Mannheim (Mannheim, Germany).

Rat mammary (Rama 27) fibroblasts (*22*) and the human benign mammary (Huma) epithelial cells, Huma 123, and the Huma 109 myoepithelial-like cells were cultured as described (*23*). The MCF-7 and ZR-75 human malignant mammary epithelial cells (*24*) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum, 50 ng/mL insulin, and 10 ng/mL oestradiol (Sigma).

Purification of HS. HS was prepared as described previously (*21*). Human mammary cells were grown in 64 15 cm culture dishes (Nunc, Denmark). To four of the dishes were added 10 μCi/mL [^{3}H]glucosamine and 20 μCi/mL [^{35}S]-SO_4 (both ICN-Flow, Thame, U.K.). When the cells were 90% confluent, the culture medium was removed and pooled with two 5 mL phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, pH 7.2) washes of the cells and then filtered through a 0.2 μm 47 mm diameter filter to remove any floating cells and cell debris. The cell monolayers were detached by scraping in 5 mL of 6 M urea/0.5% (v/v) Triton X-100, and were then incubated in this solution overnight at 4 °C on a shaker.

Two cellular samples of HS were prepared from the fibroblastic Rama 27 cells. When the cells were 90% confluent, the culture medium was collected as described for the human mammary cells. The cells were then incubated with 2 mL of 0.5% trypsin (w/v, Sigma) in versene (Gibco, Paisley, U.K.) per 15 cm dish for 10 min at 37 °C to detach the cells which were then centrifuged at 3000 rpm for 10 min in 30 mL universal tubes. The supernatant was collected, and the cell pellet was washed with 5 mL of PBS by centrifugation as above. The second supernatant was pooled with the first one to produce a fraction called the "trypsinate", containing cell surface HSPGs. The culture dishes were scraped in 5 mL of 6 M urea, 0.1% Triton X-100 in PBS, which was added to the cell pellet and solubilized as above. The culture medium and cell layer extracts were applied onto a 2.5 × 50 cm column of diethylaminoethyl (DEAE) Sepharose Fast Flow (Pharmacia, Uppsala, Sweden). Bound macromolecules were eluted with a linear gradient of NaCl (0.15−2 M NaCl in 20 mM Na_2HPO_4, pH 6.8), and the ^{3}H and ^{35}S content of aliquots of the 6 mL fractions eluting between 0.3 and 1 M NaCl, which contain proteoglycans (*25*, *26*), was determined in a Packard 1900TR scintillation counter.

The proteoglycans were dialyzed exhaustively against H_2O for 48 h and then freeze-dried. The lyophilized material was treated sequentially with (i) chondroitinase ABC, (ii) sialidases (*27*), (iii) endo-β-galactosidase and nucleases (*28*), (iv) Pronase (*27*), the latter releasing HS chains as peptidoglycans. The samples were then boiled for 3 min at 100 °C and the free HS chains fractionated on a second DEAE Sepharose fast flow column (1 × 30 cm). The HS chains were eluted with a linear gradient of 0.15−2 M NaCl in 20 mM Na_2HPO_4, pH 6.8, and were identified by measuring the ^{3}H and ^{35}S content by dual label liquid scintillation counting of aliquots of chromatography fractions. The HS chains were dialyzed exhaustively against H_2O and freeze-dried.

Biotinylation of HS. The HS peptidoglycans were biotinylated on the free amino groups that occur on the short peptide resistant to the Pronase treatment. Ten microliters of a 50 mM solution of *N*-hydroxysuccinimide aminocaproate (LC) biotin (Pierce-Warriner, Chester, U.K.) in dimethyl sulfoxide was added 3 times over 72 h to 100 μg of HS in 100 μL of distilled water. Unreacted biotin was removed by fractionation on a Sephadex G-25 column (1 × 25 cm) equilibrated in distilled water.

The percentage of the HS peptidoglycan chains possessing biotin coupled exclusively to the peptide moiety was determined by measuring the total number of binding sites for basic fibroblast growth factor (bFGF) in the HS before and after a β-elimination reaction. The total number of binding sites for bFGF on biotinylated HS immobilized on a streptavidin-derivatized aminosilane surface (*21*) was measured by repeatedly adding bFGF until saturation was achieved. After removal of the bound bFGF with 2 M NaCl, β-elimination was carried out on the immobilized HS for 16 h in 50 mM NaOH and 2 M $NaBH_4$ at 37 °C (*28*) in the biosensor cuvette. The reaction was stopped with 4 M acetic acid and the supernatant removed. A 50 μL wash of the cuvette with 8 M guanidine hydrochloride was pooled with the supernatant, which was desalted on Sephadex G-25 in H_2O. The desalted HS chains were then immobilized on a fresh streptavidin-derivatized aminosilane surface and the total number of bFGF binding sites again measured. Between 90% and 97% of the HS chains only possessed biotin which was sensitive to β-elimination, and thus linked to the peptide moiety of the peptidoglycan. The remainder of the peptidoglycan chains possessed biotin that was insensitive to β-elimination, which may be linked to the polysaccharide moiety (result not shown).

Binding Assays. Binding reactions were carried out in an IAsys resonant mirror biosensor using three-dimensional (carboxymethyl)dextran surfaces (Affinity Sensors, Saxon Hill, Cambridge, U.K.). Two distinguishable responses were observed. Rapid bulk shifts occurred within 5 s of the solution in the cuvette being changed, due to the different refractive indices of the two solutions, while sustained responses followed due to the association and dissociation of the ligate from the immobilized ligand (Figure 1A). Biotinylated HS chains were immobilized on (carboxymethyl)dextran surfaces derivatized with streptavidin according to the manufacturer's instructions. Control, streptavidin-derivatized (carboxymethyl)dextran cuvettes failed to bind HGF/SF (result not shown).

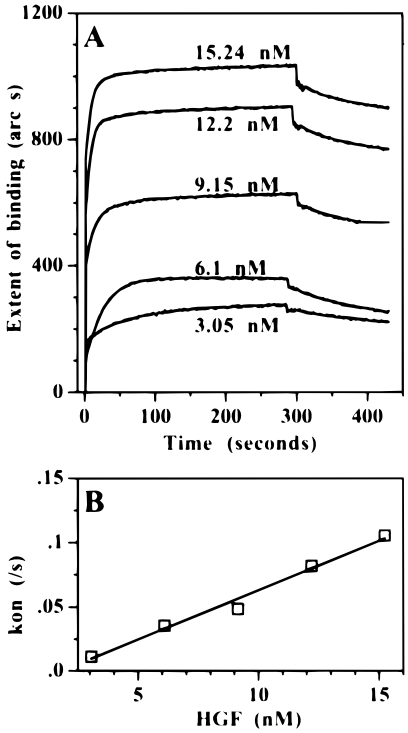

FIGURE 1: Binding of HGF/SF to HS purified from Huma 123 benign epithelial cells. HS was biotinylated and immobilized on a streptavidin−carboxymethyldextran surface (Materials and Methods). (A) Following the addition of HGF/SF to the biosensor cuvette, the binding of HGF/SF was followed for at least 5 min. The cuvette was then quickly washed 3 times with 200 μL of PBST, and the dissociation of bound HGF/SF into 200 μL of PBST was followed over the next 2 min. Data were collected every second during the course of the experiment. (B) Plot of k_{on} against ligand concentration, the slope of which is the association rate constant, k_{ass}. The k_{on} of HGF/SF for HS at each concentration of HGF/SF was determined using the FastFit software, the SE in each determination of k_{on} is smaller than the symbol.

Binding reactions were carried out at 20 °C as described (*20*, *21*). Briefly, a single binding assay consisted of adding HGF/SF at a known concentration in 100 μL of PBST [PBS supplemented with 0.02% (v/v) Tween 20] and then following the association reaction over a set time, usually 300 s. The cuvette was then washed 3 times with 200 μL of PBST, and the dissociation of bound ligate into the bulk PBST was followed over time. To remove residual bound ligate, and thus regenerate the immobilized ligand, the cuvette was washed twice with 200 μL of 2 M NaCl, 10 mM Na_2HPO_4, pH 7.2, over 2 min. This procedure removed 98−100% of the bound ligate.

A single binding assay (e.g., Figure 1A, 15.24 nM HGF/SF) yielded three binding parameters: the on-rate constant (k_{on}) and the extent of binding, calculated from the association phase, and the off-rate constant (k_{off}, equivalent to the dissociation rate constant, k_{diss}), calculated from the dissociation phase.

k_{on} and k_{off} were calculated as the change in response over time in the defined region using the nonlinear regression FastFit software (Affinity Sensors) to fit the binding data to one-site and two-site models. The likelihood of the data fitting the more complex two-site model was determined using the FastFit software, as described previously for the Ligand program (*29*). The k_{on} of a ligate for the ligand immobilized in a single cuvette was measured at a minimum of five different concentrations of ligate, and the slope of a plot of k_{on} against concentration of ligate yielded the association rate constant, k_{ass} (Figure 1B). The equilibrium dissociation constant, K_d, was then determined as the ratio of the rate constants.

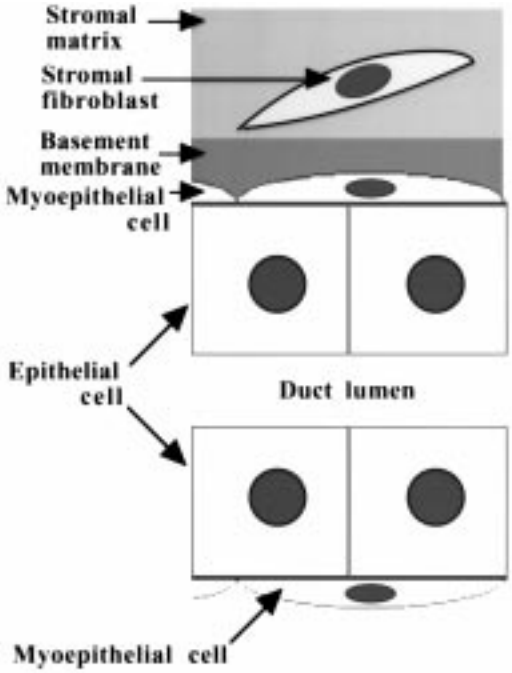

FIGURE 2: Schematic longitudinal section through a mammary duct showing the cell types in the parenchyma and the neighboring stroma. (Light gray) stromal matrix; (dark gray) basement membrane.

RESULTS

HS Chains. HS chains were purified as peptidoglycans from a panel of cell lines that are representative of some of the cell types found in the normal mammary gland (Figure 2) as well as in malignant mammary tumors. Thus, the benign Huma 123 epithelial cells correspond to the ductal epithelial cell, the Huma 109 myoepithelial-like cells possess a phenotype similar to that of the myoepithelial cells, and the Rama 27 fibroblasts are derived from mammary stromal fibroblasts, the precursor of the adipocytes of the mammary fat pad (*22*, *23*). Exhaustive digestion of the peptidoglycans with heparinases I, II, and III, followed by isolation of the disaccharides on Biogel P6, indicated that over 90% of the starting material was degraded to disaccharides and was thus HS (*30*). The composition of the disaccharides was determined in those cases where there was sufficient material. The differences observed were a 2-fold increase in 6-O-sulfated *N*-acetylglucosamine and a 4-fold increase in N-sulfated, 6-O-sulfated glucosamine in the HS from the two malignant cell lines, MCF-7 and ZR-75, compared to the HS from the benign epithelial Huma 123 and the myoepithelial-like Huma 109 cell lines. Thus, the disaccharides containing 6-O-sulfated *N*-acetylglucosamine make up 4.2% and 3.9% of the HS from the culture medium and the cell layer of the benign Huma 123 epithelial cells, respectively, but 11% and 13% of the HS from the malignant ZR-75 and MCF-7 cells, respectively. In addition, the disaccharides containing N-sulfated, 6-O-sulfated glucosamine make up 1.8% and 2.1% of the HS from the culture medium and cell

Table 1: Extent of Binding of 24.4 nM HGF/SF to HS Isolated from Mammary Cells and k_{off} of the Subsequent Dissociation

source of HS	extent of binding[a] (arc s)	k_{off} (s^{-1})
Rama 27 fibroblasts		
culture medium	43	ne[b]
cell surface	56	0.0047 ± 0.0033
extracellular matrix	40	0.0094 ± 0.0023
Huma 123 benign epithelial cells		
medium	160	0.00041 ± 0.00036
cells	650	0.0075 ± 0.0014
Huma 109 myoepithelial-like cells		
medium	62	0.0082 ± 0.0018
cells	82	0.0069 ± 0.002
MCF-7 malignant epithelial cells		
medium	260	0.0066 ± 0.0007
cells	320	0.00049 ± 0.00032
ZR-75 malignant epithelial cells		
medium	nd[c]	nd[c]
cells	460	0.0097 ± 0.0004

[a] Results are the extent of binding of 2 ng/μL (24.4 nM) HGF/SF to HS immobilized on a (carboxymethyl)dextran surface (Materials and Methods). The SE was less than 2% of the value. [b] ne: no evidence for dissociation of the HS−HGF/SF complex. [c] nd: not determined.

layer of Huma 123 cells, but 8.9% and 11% of the HS from the malignant ZR-75 and MCF-7 cells (*30*, *31*).

Relative Levels of Binding of HGF/SF to HS. An initial assessment of the binding of 2 ng/μl (24.2 nM) HGF/SF to the HS isolated from the different mammary cell lines was performed. Changes in the refractive index after the bulk shift are reported as a response in arc seconds (1 arc s = 1/3600°), and for proteins a response of 163 arc s is equivalent to 1 ng of protein bound to 1 mm^2 of the cuvette surface (*32*, *33*). The results indicated that the highest levels of binding of HGF/SF (160−750 arc s) were observed with the cellular and medium HS isolated from the three epithelial cell lines: benign Huma 123, and malignant MCF-7 and ZR-75. In contrast, the medium and cellular HS from the Huma 109 myoepithelial-like cells, and the fibroblastic Rama 27 cells, bound HGF/SF at this concentration to a lesser extent (43−85 arc s) (Table 1). The k_{off} was also determined in these experiments. k_{off} is equivalent to the dissociation rate constant, and despite these measurements representing a single determination of this binding parameter, the different samples of HS clearly possess different dissociation rate constants. k_{off} could not be determined for HGF/SF bound to the HS from the culture medium of the Rama 27 fibroblasts (Table 1), which could either be a genuine observation or be an artifact due to the low level of binding during the association phase of the reaction. HGF/SF dissociated slowly from the HS isolated from the MCF-7 malignant cells and the culture medium of the benign Huma 123 cells but about 10-fold faster from the other samples of HS (Table 1).

Kinetics of Binding of HGF/SF to HS from Parenchymal Cells. A full characterization of the binding parameters of HGF was performed on the HS isolated from the cells originating from the mammary parenchyma and on the HS from the culture medium of the Huma 123 benign epithelial cells, since these samples possessed different values for k_{off} (Table 1). The association rate constant (k_{ass}) of HGF/SF for the cellular HS from the Huma 123 epithelial cells (Figure 1), the Huma 109 myoepithelial-like cells, and the malignant epithelial ZR-75 cells was above 10^6 M^{-1} s^{-1} and was termed "fast" (Table 2). The dissociation rate constant of HGF/SF from the same samples of HS was also fast (Figure 1 and Table 2), and these values are representative of the faster rate of dissociation noted earlier (Table 1). Therefore, the binding site for HGF/SF on the HS from the Huma 123, Huma 109, and ZR-75 cells is termed "fast-fast". While the association rate constant of HGF/SF for the HS from the culture medium of the Huma 123 cells was fast, the dissociation rate constant of HGF/SF from this species of HS was slow (Table 2), thus forming a "fast-slow" binding site. The HS isolated from the malignant MCF-7 epithelial cells had an association rate constant for HGF/SF that was 7−38-fold slower than that observed for the other cell lines (Table 2), and a dissociation rate constant of HGF/SF for this species of HS was similar to the slow k_{diss} observed with the HS from the culture medium of the Huma 123 cells (Table 2). The HS from the MCF-7 cells thus has "slow-slow" HGF/SF binding kinetics.

The K_ds for HGF/SF of the "fast-fast" and of the "slow-slow" binding sites on the samples of cellular HS were similar, ranging from 1 to 3 nM (Table 2), and would thus not be distinguishable in equilibrium binding assays. In contrast, since the HS from the culture medium of the Huma 123 cells possessed "fast-slow" HGF/SF binding kinetics, its K_d for HGF/SF is considerably lower, 200 pM.

DISCUSSION

Distinct Potential Binding Sites for HGF/SF in HS. As a consequence of the different association and dissociation rate constants of the samples of cellular HS, there are at least three kinetically distinct binding sites for HGF/SF (Table 2).

One site, present on the HS purified from the Huma 123 benign epithelial cells, the Huma 109 myoepithelial-like cells, and the ZR-75 malignant epithelial cells, possesses fast association and dissociation rate constants, thus exhibiting "fast-fast" binding kinetics and a K_d of 1−3 nM.

The second site, present on the HS from the MCF-7 malignant epithelial cells possesses slower association and dissociation rate constants and thus has "slow-slow" binding kinetics and a K_d similar to the "fast-fast" site.

The third site, present on the HS from the culture medium of the Huma 123 epithelial cells, has "fast-slow" binding kinetics and a K_d of 200 pM, which is comparable to that of a site described previously (*34*).

The group of "fast" association rate constants ($k_{ass} > 10^6$ M^{-1} s^{-1}) may be subdivided into two categories: "medium", ranging from 1.1 to 1.4 × 10^6 M^{-1} s^{-1}; and "fast", >7 × 10^6 M^{-1} s^{-1}. Whether the grouping of these rate constants into a single class of "fast" sites is justified will require the determination of the structures in HS which underlie each set of HGF/SF binding kinetics.

The three binding sites seem to be mutually exclusive, since in the course of the analysis of the binding data, it was apparent that the binding sites for HGF/SF in each sample of HS were remarkably homogeneous; i.e., there was no evidence for the presence of two or more binding sites in a single sample of HS (Table 2). Each sample of HS chains used in this study represents a population of molecules of the same type but which may possess different fine structures and thus protein-binding sequences. Therefore,

Table 2: Kinetics of HGF/SF Binding to Immobilized HS Purified from Mammary Cells

source of HS	k_{ass} (M^{-1} s^{-1})[a] × 10^6	r[b]	k_{diss} (s^{-1})[c]	K_d (nM)[d]	type of binding site
Huma 123 benign epithelial	7.7 ± 0.06	0.99	0.0096 ± 0.0065	1.3 ± 0.1	"fast-fast"
Huma 123 benign epithelial culture medium	1.10 ± 0.15	0.96	0.00021 ± 0.00008	0.20 ± 0.08	"fast-slow"
Huma 109 myoepithelial-like	1.40 ± 0.37	0.94	0.0032 ± 0.0017	2.5 ± 1.4	"fast-fast"
MCF-7 malignant epithelial	0.20 ± 0.05	0.95	0.00055 ± 0.00018	2.8 ± 1.2	"slow-slow"
ZR-75 malignant epithelial	7.70 ± 0.06	0.99	0.0095 ± 0.0040	1.2 ± 0.5	"fast-fast"

[a] The SE is derived from the deviation of the data from a one-site binding model, calculated by matrix inversion using the FastFit software provided with the instrument (Materials and Methods). No evidence was found for a two-site model of association, and so the HGF/SF-binding sites in each sample of HS were homogeneous in this respect. Since at least two sets of values for k_{on} were obtained, the resulting values for the calculated k_{ass} and its associated error were combined. [b] The correlation coefficient of the linear regression through the k_{on} values for the determination of k_{ass}. [c] The k_{diss} is the mean ± SE of at least four values, obtained at different concentrations of HGF/SF. No evidence was found for a two-site model of dissociation, and so the HGF/SF-binding sites in each sample of HS were homogeneous in this respect. [d] The K_d was calculated from the ratio of k_{diss}/k_{ass}, and the SE is the combined SE of the two kinetic parameters.

the presence of only one binding site for HGF/SF in each sample of HS studied suggests that at least these mammary cells synthesize HS destined for a particular location (cellular or culture medium) with "fast-fast", "slow-slow", or "fast-slow" binding kinetics for HGF/SF, but not a combination of these binding sites. For example, the Huma 123 epithelial cells are able to synthesize HS destined for the culture medium with "fast-slow" HGF/SF binding kinetics, while HS destined for the cell layer possesses "fast-fast" HGF/SF binding kinetics. The results, however, give no information on the number of binding sites present on each HS chain.

Implications for the Structure of Mammary Cell HS. A binding structure for HGF/SF in HS isolated from stromal cells, human fetal skin fibroblasts, has been characterized (*11*). The minimal binding sequence seems to be a hexasaccharide, although decasaccharides have the highest affinity for HGF/SF, as defined by the concentration of NaCl required to dissociate the HGF/SF−HS complex. The key features of this binding structure are the presence of 6-*O*-sulfate groups on N-sulfated glucosamine and the presence of iduronic acid. Although the *N*-sulfate group of the glucosamine and to a large extent the 2-*O*-sulfate group on the adjacent iduronic acid seemed to play little role in binding (*11*), it is only within N-sulfated sequences that high concentrations of iduronate residues occur; 6-*O*-sulfates also occur in these N-sulfated domains and in the flanking regions (*35*, *36*). The importance of the 6-*O*-sulfate group on the glucosamine for the binding of HGF/SF has subsequently been confirmed, as well as the relative unimportance of 2-*O*-sulfate groups on iduronic acid (*37*). The present results therefore suggest that the HS from the cells derived from the mammary epithelium contains reasonably large contiguous stretches of 6-*O*-sulfated glucosamine. The major differences in the composition of the HS chains from the different mammary cell lines are an overall 2-fold increase in the content of 6-*O*-sulfate-containing disaccharides in HS isolated from the malignant MCF-7 and ZR-75 cells, compared to the HS from the Huma 123 epithelial cells and the Huma 109 myoepithelial-like cells (*30*, *31*). However, this change in content of 6-*O*-sulfate does not correlate with changes in the HGF/SF-binding properties of the different species of HS. Moreover, the HS from the culture medium and the cell layer of the Huma 123 cells possess "fast-slow" and "fast-fast" HGF/SF binding kinetics, respectively, (Table 2), yet have the same content of 6-O-sulfated glucosamine, 6% (*30*, *31*). Therefore, differences in the fine structure of HGF/SF-binding sequences, e.g., their length and/or the disposition of sulfate groups, are more likely to be the structural explanation for the three kinetically distinct binding sites for HGF/SF rather than differences in overall HS chain composition.

Biological Implications of the Three Binding Sites for HGF/SF in Mammary Cell HS. The three types of binding site on HS identified in the present study are likely to interact differently with HGF/SF. The "fast-fast" site will allow efficient capture and release of HGF/SF and thereby facilitate diffusion of the growth factor. The "slow-slow" site, and in particular the "fast-slow" site, will tend to retain the HGF/SF, restricting its diffusion. If the HS produced by epithelial, myoepithelial, and fibroblastic cells in vivo possessed the same pattern of HGF/SF-binding sites as the HS isolated from the cell culture model, the diffusion of HGF/SF would be directional. Thus, HGF/SF, which is produced by the cells of the mammary stroma (*16*), would diffuse from the stromal cells through the basement membrane (deposited by stromal fibroblasts and myoepithelial cells (*14*)) and the underlying layer of myoepithelial cells to the lumenal epithelial cells, which possess both the *met* tyrosine kinase receptor and "fast-slow" HS receptors for HGF/SF.

Cells from malignant mammary tumors invade the surrounding stroma by breaking down the basement membrane (*14*). The "fast-fast" and especially the "slow-slow" receptors found on the malignant epithelial cells (Table 2) would allow such cells to capture HGF/SF released from stromal HS and basement membrane HS during the invasion.

A second potential regulatory function for the three binding sites of HGF/SF in mammary cell HS is the possibility of their modulating the interaction between HGF/SF and the *met* tyrosine kinase receptor. Thus, activation of HGF/SF by the "slow-slow" and "fast-slow" HS receptors could enable a prolonged interaction with HGF/SF, resulting in chronic activation of the *met* tyrosine kinase, while the "fast-fast" HS receptor could result in a more transient activation of the *met* tyrosine kinase.

Therefore, the activity of stromal HGF/SF on the mammary parenchyma may be regulated by the type of HS receptors present on stromal cells, in the basement membrane, and on the cells of the mammary parenchyma itself. The HGF/SF-binding characteristics of these HS receptors would appear to depend on the fine structure of the HS rather than the overall composition of the HS chains, and a clear challenge for the future is to identify the structures within the mammary HS chains that are responsible for the three HGF/SF-binding sites revealed by the biosensor-based binding assays.

REFERENCES

1. Gallagher, J. T. (1989) *Curr. Opin. Cell Biol. 1*, 1201−1218.
2. Kjé llen, L., and Lindahl, U. (1991) *Annu. Rev. Biochem. 60*, 443−475.
3. Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L., and Lose, E. J. (1992) *Annu. Rev. Cell Biol. 8*, 365−393.
4. Elenius, K., and Jalkanen, M. (1994) *J. Cell Sci. 107*, 2975−2982.
5. Yanagishita, M., and Hascall, V. C. (1992) *J. Biol. Chem. 267*, 9451−9454.
6. Rudland, P. S., Platt-Higgins, A. M., Wilkinson, M. C., and Fernig, D. G. (1993) *J. Histochem. Cytochem. 41*, 887−898.
7. Richardson, L., Schwall, R. H., and Zioncheck, T. F. (1996) *Trends Glycosci. Glycotechnol. 8*, 167−182.
8. Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) *Science 252*, 1705−1708.
9. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell 64*, 841−848.
10. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) *J. Biol. Chem. 269*, 931−935.
11. Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T., and Gallagher, J. T. (1994) *J. Biol. Chem. 269*, 11216−11223.
12. Sakata, H., Stahl, S. J., Taylor, W. G., Rosenberg, J. M., Sakaguchi, R., Wingfield, P. T., and Rubin, J. S. (1997) *J. Biol. Chem. 272*, 9457−9463.
13. Schwall, R. H., Chang, L. Y., Godowski, P. J., Kahn, D. W., Hillan, K. J., Bauer, K. D., and Zioncheck, T. F. (1996) *J. Cell Biol. 133*, 709−718.
14. Rudland, P. S., Barraclough, R., Fernig, D. G., and Smith, J. A. (1996) in *Stem cells and cancer* (Potton, C., Ed.) pp 147−232, Churchill Livingstone, London, U.K.
15. Rudland, P. S., Fernig, D. G., Leinster, S., and Lunt, G. G. (1997) in *Biochem. Soc. Symp. 63*, Portland Press, Colchester, U.K.
16. Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K. M., and Birchmeier, W. (1995) *J. Cell Biol. 131*, 1573−1586.
17. Emerman, J. T., and Pitelka, D. R. (1977) *In Vitro 13*, 313−328.
18. Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L., and Montesano, R. (1995) *J. Cell Sci. 108*, 413−430.
19. Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. (1995) *Development 121*, 2897−2908.
20. Kinsella, L., Chen, H.-L., Smith, J. A., Rudland, P. S., and Fernig, D. G. (1998) *Glycoconjugate J. 15*, (in press).
21. Rahmoune, H., Chen, H.-L., Gallagher, J. T., Rudland, P. S., and Fernig, D. G. (1998) *J. Biol. Chem. 273*, 7303−7310.
22. Rudland, P. S., Twiston Davis, A. C., and Tsao, S. W. (1984) *J. Cell. Physiol. 120*, 364−376.
23. Rudland, P. S. (1993) *Histol. Histopathol. 8*, 385−404.
24. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. (1973) *J. Natl. Cancer Inst. 51*, 1409−1413.
25. Turnbull, J. E., and Gallagher, J. T. (1991) *Biochem. J. 273*, 553−559.
26. Turnbull, J. E., and Gallagher, J. T. (1991) *Biochem. J. 277*, 297−303.
27. Turnbull, J. E., and Gallagher, J. T. (1990) *Biochem. J. 265*, 715−724.
28. Rahmoune, H., Rounding, H. P., McDonald-Gibson, W. J., Lamblin, G., Hall, R. L., and Roussel, R. (1991) *Am. J. Respir. Cell Mol. Biol 4*, 156−165.
29. Fernig, D. G., Rudland, P. S., and Smith, J. A. (1992) *Growth Factors 5*, 27−39.
30. Rahmoune, H., Turnbull, J. E., Gallagher, J. T., Rudland, P. S., and Fernig, D. G. (1996) *Biochem. Soc. Trans. 24*, 355.
31. Rahmoune, H., Turnbull, J. E., Gallagher, J. T., Rudland, P. S., and Fernig, D. G. (1997) *Biochem. Soc. Trans. 25*, 1299.
32. Cush, R., Cronin, J. M., Stewart, W. J., Maule, C. H., Molloy, J. O., and Goddard, N. J. (1993) *Biosens. Bioelectron. 8*, 355−363.
33. Yeung, D., Gill, A., Maule, C. H., and Davies, R. J. (1995) *Trends Anal. Chem. 14*, 49−55.
34. Tajima, H., Matsumoto, K., and Nakamura, T. (1992) *Exp. Cell Res. 202*, 423−431.
35. Maccarana, M., Sakara, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem. 271*, 17804−17810.
36. Sanderson, P. N., Huckerby, T. N., and Nieduszynski, I. A. (1984) *Biochem. J. 223*, 495−505.
37. Ashikari, S., Habuchi, H., and Kimata, K. (1995) *J. Biol. Chem. 270*, 29586−29593.

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