A role for the perlecan protein core in the activation of the keratinocyte growth factor receptor

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Perlecan, a widespread heparan sulphate (HS) proteoglycan, is directly involved in the storing of angiogenic growth factors, mostly members of the fibroblast growth factor (FGF) gene family. We have previously shown that antisense targeting of the perlecan gene causes a reduced growth and responsiveness to FGF7 [also known as keratinocyte growth factor (KGF)] in human cancer cells, and that the perlecan protein core interacts specifically with FGF7. In the present paper, we have investigated human colon carcinoma cells in which the perlecan gene was disrupted by targeted homologous recombination. After screening over 1000 clones, we obtained two clones heterozygous for the null mutation with no detectable perlecan, indicating that the other allele was non-functioning. The perlecan-deficient cells grew more slowly, did not respond to FGF7 with or without the addition of heparin, and were less tumorigenic than control cells. Paradoxically, the perlecan-deficient cells displayed increased FGF7 surface binding. However, the perlecan protein core was required for functional activation of the KGF receptor and downstream signalling. Because heparin could not substitute for perlecan, the HS chains are not critical for FGF7-mediated signalling in this cell system. These results provide the first genetic evidence that the perlecan protein core is a molecular entity implicated in FGF7 binding and activation of its receptor.

Key words: fibroblast growth factor, FGF7, FGF7 receptor, heparan sulphate proteoglycan, somatic gene targeting.

INTRODUCTION

The fibroblast growth factor (FGF) family plays an important role in a variety of physiological and pathological processes [1,2]. FGFs interact predominantly with two cell surface receptors: a family of tyrosine kinase receptors [FGF receptors (FGFRs)] that mediate the cell signalling activities of FGFs [3], and heparan sulphate (HS) proteoglycans (HSPGs) that act as co-receptors [4]. Four closely-related FGFRs (FGFR1–FGFR4) have been isolated, and an additional level of complexity is created by alternative splicing that generates FGFR isoforms with altered ligand binding properties [1]. The members of the FGF family bind FGFRs with varying affinities [5]. For example, FGF1 binds with high affinity to the four known receptors and all the isoforms that have been thus far investigated, whereas FGF2 binds FGFR1, FGFR2 and FGFR4 with high affinity. Unlike the other members of the FGF family, FGF7 [also known as keratinocyte growth factor (KGF)] [6,7] recognizes only one FGFR isoform [5], the type 2(IIIb) FGFR, a splice variant of FGFR2. FGF7 is secreted by cells of mesenchymal origin and acts in a paracrine fashion on nearby epithelial cells that express FGFR2b [1]. FGF7 may play a specialized role in tumorigenesis, since most human malignancies arise in epithelial tissues where cell populations are continuously turning over [7].

In addition to binding to their transducing receptors, FGFs also bind to heparin and to the HS chains of proteoglycans [8–10]. Unlike heparin, which is primarily located intracellularly and is exclusively synthesized in mast cells, HS expression is ubiquitous and is abundant at the cell surface and in the extracellular matrix [11,12]. These low-affinity, but high-capacity, binding sites have considerable biological significance and it is now well established that HS and heparin are potent modulators of FGF activity [13–15]. The physiological roles of HS include protection from thermal denaturation and proteolytic degradation of the bound mitogen, and the formation of a bioactive reservoir from which FGFs can be rapidly released in response to specific triggering events [16–18]. In the absence of cell surface HS, cellular responses to FGFs are attenuated but can be restored by the addition of heparin [13,14]. In particular it has been shown that HS increases the binding efficiency of FGFs and FGFR dimerization, which is necessary for receptor phosphorylation [19]. Because the ability of cells to synthesize HS side chains depends on the level of expression of various proteoglycan protein cores [20–22], their identification is necessary to understand the mechanisms underlying the FGF mode of action. The core protein may influence the structure and availability of the attached glycosaminoglycans and may be directly involved in the interaction between a growth factor and its receptor [23].

Perlecan, a ubiquitous HSPG [24–26], has been identified as a major candidate for the FGF2 low-affinity accessory receptor, and is sufficient to restore high-affinity binding of FGF2 to the tyrosine kinase receptor in HS-deficient cells [27]. Disruption of the perlecan gene in mice causes embryonic lethality at day 10.5 with severe cephalic and cartilaginous abnormalities [28,29]. Although basement membrane structures can develop in the absence of perlecan, most of the animals succumb to intrapericardial haemorrhages at a time when vasculogenesis is prominent and intraventricular pressure rises [29], implicating perlecan in the maintenance of basement membrane integrity. In

Abbreviations used: BS^3, bis(sulphosuccinimidyl) suberate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; FCS, fetal calf serum; FGF, fibroblast growth factor; FGFR, FGF receptor; HS, heparan sulphate; HSPG, HS proteoglycan; KGF, keratinocyte growth factor; KGFR, KGF receptor; MAPK, mitogen-activated protein kinase; tk, thymidine kinase.

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addition, the cartilage phenotype of the perlecan-deficient animals that reach adulthood resembles that caused by activating mutations of FGFR3, indicating that perlecan may act as a negative modulator of FGFR3, and thus positioning perlecan in this signalling transducing pathway [28].

Inhibition of endogenous perlecan levels in human melanoma cells suppresses autocrine and paracrine functions of FGF2 and blocks melanoma cell proliferation and invasion [30,31]. Likewise, antisense targeting of the perlecan gene causes a marked attenuation of the growth of colon carcinoma cells [32]. These effects correlate with a reduced mitogenic response to FGF7 and with the efficient reconstitution of FGF7 activity by supplementation of purified perlecan. Using overlay protein assays, radioligand binding experiments and the yeast two-hybrid system we demonstrated that FGF7 binds specifically to the N-terminal half of domain III and to a lesser extent to domain V, with affinity constants in the range of 60 nM [33]. Thus the perlecan protein core should be considered a novel biological ligand for FGF7.

The present study was undertaken to investigate the role of perlecan in FGF7/KGF receptor (KGFR)-mediated signal transduction. For this purpose, a perlecan-deficient human colon carcinoma cell line (HCT116) was generated through somatic gene targeting and clonal selection. By comparing the responsiveness to FGF7 of the modified cell line with the wild-type parental cell line, we conclude that perlecan directly participates in FGF7-mediated activation of KGFR. The signal transduction functionality in perlecan-deficient cells could not be rescued by heparin, consistent with the concept that the perlecan core protein, rather than the HS chains, is the molecular entity implicated in FGF7 signalling.

MATERIALS AND METHODS

Materials and cells

Human recombinant FGF1, FGF7 and epidermal growth factor (EGF) were purchased from R & D Systems (Minneapolis, MN, U.S.A.). Media and fetal calf serum (FCS) were obtained from Hyclone Laboratories (Logan, UT, U.S.A.). ^{125}I and Hybond ECL® membranes were purchased from Amersham. Bis(sulphosuccinimidyl) suberate (BS^3), a protein cross-linker, and Iodo-Gen™ were bought from Pierce. The secondary antibodies were obtained from Sigma, and the protease inhibitors (aprotinin, leupeptin, bestatin and PMSF) were from Boehringer Mannheim. Monoclonal antibodies raised against domain III of perlecan [34], the intracellular domain of KFGR/FGFR2 [anti-Bek monoclonal antibody (where Bek is an alternative name for KGFR); C8; Santa Cruz Biotechnology] and phosphotyrosine (PY20; Transduction Laboratories) were used. Anti-[phospho-p44/42 mitogen-activated protein kinase (MAPK)] was purchased from New England Biolabs. Human colon carcinoma HCT116 and WiDr, HeLa and fibrosarcoma HT1080 cells were obtained from A.T.C.C. (Rockville, MD, U.S.A.).

Construction of the promoterless perlecan targeting vector and clonal selection

For the construction of the promoterless targeting vector, a human *Sma*I 5.5 kb genomic fragment harbouring exons 2 and 3 was retrieved from a plasmid derived from a specific human chromosome 1 cosmid library [35]. The insert was subcloned in pBS (ClonTech Laboratories) at the same restriction sites. Exon 2, which contains a unique *Nco*I restriction site, was selected for the insertion of the *Neo* selection cassette or alternatively the *Hygro* selection cassette. The promoterless *Neo* cassette was retrieved from the pSV-*Neo* plasmid by digestion with *Eag*I and *BamH*I, ligated in pBS and retrieved by digestion with *BamH*I and *Sac*I. This insert was then ligated at the blunt-ended *Nco*I site of exon 2, thus generating a transcript comprising exons 1 and 2 of perlecan and the promoterless *Neo* gene including a stop codon and a polyadenylation signal. For the generation of the second targeting vector, the promoterless *Hygro* cassette was amplified from the pSVtk-*Hygro* vector (where tk stands for thymidine kinase) by PCR using modified primers to generate terminal *Eco*RV restriction sites. The cassette was then ligated at the blunt-ended *Nco*I sites of exon 2. In-frame ligations were confirmed by direct sequencing. The targeting vectors were cloned in *rec*A1 mutated *Escherichia coli* bacteria (DH5α) and purified by ion-exchange chromatography. The vectors were linearized by digestion with *Xho*I at a site flanking the 5′ end of the region of homology, and recovered by phenol/chloroform extraction and ethanol precipitation. The resulting DNA was solubilized in water and 50 μg was transfected into the cells by electroporation at 230 V and 1080 μF (at 4 °C). Transfected cells were replated in 10-cm plastic dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) FCS for 2 days, followed by selection in G418 (400–600 μg/ml). Within 2 weeks after electroporation the drug-resistant colonies were ring-cloned and expanded. The occurrence of homologous recombination of the targeting vector at the perlecan locus was verified by Southern hybridization using a ^{32}P-labelled probe spanning the 1.4 kb flanking region 3′ to the targeting vector. For this purpose, cell DNA (approx. 10 μg) was digested with *Nco*I and electrophoresed on a 0.7 % agarose gel followed by transfer on to nitrocellulose. Filters were hybridized with the ^{32}P-labelled probe under stringent conditions and exposed for autoradiography. Targeting of the second allele was pursued by performing a second round of transfection with the promoterless *Hygro* cassette described above. For selection, cells were grown in the presence of 150–300 μg/ml hygromycin. After genotyping of the rescued clones by Southern hybridization, final confirmation of the functional ablation of the perlecan gene was sought through Northern- and Western-blot analyses.

Northern-blot analysis and slot immunoblotting

Total RNA was extracted with Tri-Reagent (Sigma) and 20 μg was electrophoresed on 1 % (w/v) agarose gels containing 6 % (v/v) formaldehyde. Northern-blot analysis of RNA was performed as previously described [32]. A cDNA ^{32}P-probe corresponding to domain III of perlecan was used for hybridization and perlecan mRNA detection. For immunoblotting, HCT116 clones were grown to confluence in 6-well dishes. After preincubation for 12 h in serum-free medium, cells were washed and incubated for an additional 24 h in the same medium. Increasing volumes of the conditioned medium were slot-blotted on to nitrocellulose filters in Tris-buffered saline and reacted with monoclonal antibody 7B5, which recognizes domain III of human perlecan [34]. After extensive washing, the filters were incubated with rabbit anti-mouse IgG horseradish peroxidase-conjugated antibodies, and the reaction product was detected by enhanced chemiluminescence.

Responsiveness to growth factors and affinity cross-linking experiments

Cells were seeded in 48-well plates and incubated for 36 h in serum-free medium. The cells were then supplemented with FGF7. Heparin was added in some experiments (as described in the Results section). At the end of the experiments, a colorimetric

assay was performed (Cell Titer 96; Promega) where the amount of the reaction product was proportional to the number of viable cells. Alternatively, cell number was determined by counting with a microcytometer chamber. ^{125}I-FGF7 was generated by modification of the radio-iodination Bolton–Hunter procedure using Iodo-Gen™ pre-coated tubes (Pierce). For the binding experiments, cells were plated in 6-well culture plates in DMEM containing 10% (v/v) FCS and allowed to reach confluence. The medium was then replaced with 199 medium (without serum) and the cells were used 24 h later. On the day of the experiment, the cells were washed with ice-cold binding buffer (DMEM, 15 mM Hepes and 0.05% gelatin, pH 7.4) and incubated with ^{125}I-FGF7 at the indicated concentrations. The cells were washed three times with ice-cold binding buffer and the proteoglycan-bound ^{125}I-FGF was removed with a rapid wash (approx. 10 s) using 20 mM Hepes/1 M NaCl (pH 7.0). Cell surface receptor-bound ^{125}I-FGF was then extracted with two washes in 10 mM sodium acetate/1 M NaCl (pH 5.0). Confluent cells in 35-mm dishes were serum starved for 36 h, and incubated for 4 h in 1 ml of DMEM together with ^{125}I-FGF7 and increasing concentrations of heparin at 4 °C. Ligand–receptor cross-linking reactions were performed at 25 °C by addition of 50 μl of 20 mM BS^3. The reaction was terminated 20 min later by the addition of 1 M Tris (pH 7.5), and the cells were solubilized in Tris buffer containing 0.1% Triton X-100 and a cocktail of protease inhibitors. After the protein assay, cell extracts were separated by SDS/PAGE [7% (w/v) polyacrylamide] and the cross-linked products were visualized by autoradiography.

Western immunoblotting and *in vivo* studies

Confluent cells on 35-mm dishes were preincubated for 36 h in serum-free medium. On the day of the experiments, the cells received fresh DMEM and, after 3 h, FGF7 (20 and 100 ng/ml) or FGF1 (100 ng/ml) in the absence or presence of 1 μg/ml heparin was added. After incubation for 15 min at 37 °C, cells were placed on ice and washed twice with ice-cold PBS. Following cell solubilization and protein assays, the cell extracts were separated by SDS/PAGE [7% (w/v) polyacrylamide]. KGFR and MAPK phosphorylation products were examined by Western immunoblotting. Briefly, after protein transfer on to nitrocellulose, the filter was cut at the 97 kDa molecular mass standard mark. The top portion of the filter was processed for detection of phosphoproteins by incubation with monoclonal antibodies against phosphotyrosine or with anti-KGFR antibodies. The bottom part of the filter was processed for the detection of MAPK-phosphorylated products using a mouse monoclonal anti-(MAPK phosphoprotein) antibody and detection with a rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibody.

Tumour xenografts were generated by injecting approx. 10^6 wild-type or mutant cells into the subcutaneous region of immunocompromised (*nu/nu*) mice (Harlan, Indianapolis, IN, U.S.A.). Two independent experiments were performed with similar results using a total of 38 animals. Mice were carefully examined every 2 or 3 days for up to 24–30 days post-injection and any tumour growth was measured with a microcaliper using the formula $V = a(b^2/2)$, where a is the width at the widest point and b is the width perpendicular to a. At the end of the experiment, approx. 4 weeks post-injection, the animals were killed and full necropsies were performed. Tumour samples were analysed by conventional light microscopy [36]. Animals were maintained in accordance with the guidelines of the institution's Animal Care and Use Committee.

RESULTS

Targeted disruption of the perlecan gene in human colon carcinoma cells

Human colon carcinoma HCT116 cells were selected for somatic cell knockout of the perlecan gene using non-isogenic constructs. This cell line offers some key advantages over other normal or malignant cell lines. First, HCT116 cells are deficient in DNA mismatch repair thereby enhancing the stability of the inserted foreign DNA [37]. Secondly, they are sensitive to the cytotoxic effect of G418 over a relatively wide range of concentrations (between 0.4 and 10 mg/ml), which is useful when attempting to improve the rate of recovery of successful transfectants by increasing the drug concentration [38,39]. Thirdly, unlike most transformed cell lines, HCT116 cells have a normal karyotype, which implies that somatic knockout might be accomplished in two rounds of gene targeting [40]. Fourthly, these cells have been successfully used for somatic cell knockout studies of p21 [38,39,41]. Initial gene targeting experiments were performed on HCT116 as well as WiDr/HT29 and HT1080 cells. A targeting vector harbouring a cytomegalovirus-driven *Neo* cassette inserted in exon 2 with the purpose of disrupting perlecan gene processing and acting as a positive selection marker was used for this purpose. Approx. 1300 G418-resistant clones were generated and 960 were genotyped by Southern hybridization to determine whether homologous recombination had taken place (Table 1). Because no homologous recombinant clones could be rescued with this strategy a new one was attempted. This was based on the use of a promoterless targeting vector (Figure 1A) [42]. The rationale for this strategy is that expression of the selectable marker is restricted to the case of recombination of the targeting vector in areas of the genome under control of an active promoter. This effectively reduces the number of false positives, enabling a more efficient screening of the G418-resistant clones. Successful homologous recombination was achieved in two clones, as evidenced by the generation of a discriminating 4.4 kb *Nco*I restriction fragment (Figure 1B). One of the two clones, clone 76, was expanded and subjected to a new round of transfection with the *Hygro* promoterless vector to target the other perlecan allele. None of the new 83 generated hygromycin-resistant clones had a DNA restriction pattern consistent with the event of a new homologous recombination (results not shown). It was observed that approx. 10% of the hygromycin-resistant colonies duplicated at a slow rate. Because a reduced rate of growth had been observed in HCT116 cells expressing a perlecan antisense construct, and this correlated with a low level of secreted perlecan [32], we considered the possibility that the screening for perlecan knockout colonies might be negatively biased due to the slow rate of growth of these cells. To bypass this potential drawback, we began screening for perlecan secretion using hygromycin-resistant clones at the first passage following ring cloning. By doing so we identified several clones with decreased perlecan expression and two clones (clones 6 and 9/4) whose perlecan biosynthesis was below detection when assessed by slot immunoblotting of conditioned medium (Figure 1C). Northern hybridization using a perlecan-specific cDNA probe revealed a faint signal for perlecan mRNA in the perlecan-deficient cells (results not shown), suggesting that perlecan mRNA is unstable and destined for degradation in these clones.

Perlecan expression is required for proper growth and responsiveness to FGF7

To assess the role of perlecan deficiency, we cultured wild-type or various mutants in the presence and absence of 0.2% BSA or

Table 1 Perlecan gene targeting events in various tumour cell lines

Targeting vectors, either harbouring a *Pgk*-driven *Neo* selectable marker (positive/negative vector) or promotorless, were transfected by electroporation into approx. 3×10^7 cells. After 2 days of recovery, the selection was initiated by the addition of G418 or hygromycin at the indicated concentrations of the active drug. Following at least 4 weeks in the selection medium, colonies were isolated by ring cloning, and further expanded for genetic and functional characterization.

Cell line	Selection strategy	Selection marker	Drug dose (g/ml)	Subclones (*n*)	Targeted clones (*n*)
WiDr*	positive/negative	*Neo*	600	182	0
HT1080†	positive/negative	*Neo*	600	200	0
HCT116‡	positive/negative	*Neo*	600	578	0
HCT116	promotorless	*Neo*	600	43	2
HCT116	promotorless	*Hygro*	200	41	0

* Human colon carcinoma cells that synthesize high levels of human perlecan [62].
† Human fibrosarcoma previously used for stable transfection studies [63].
‡ Human colon carcinoma cells deficient in the mismatch repair gene, which have been previously used for somatic gene targeting [38–40].

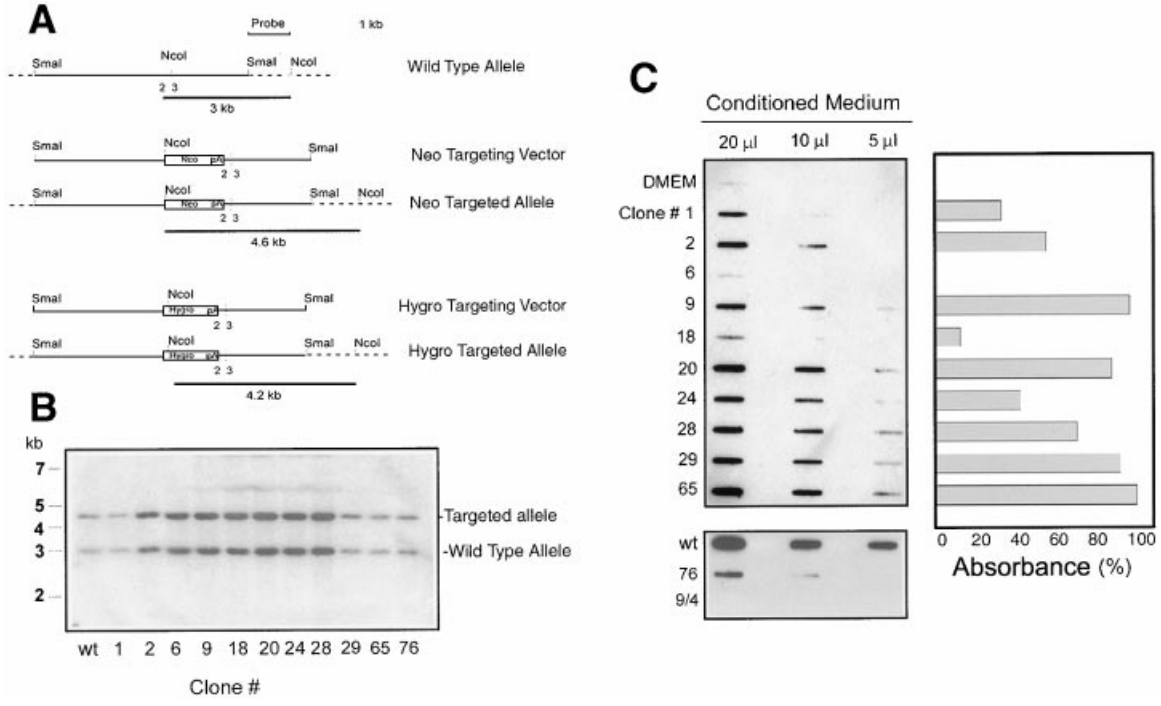

Figure 1 Somatic cell knockout of the human perlecan gene

(**A**) Targeting vector strategy and characterization of the homologous recombinant clones. Targeting vectors were generated by insertion of a promoterless *Neo* cassette into the *Nco*I site of exon 2 in a 5.5 kb genomic *Sma*I restriction fragment. The second targeting vector harbouring the *Hygro* cassette was generated with the same strategy. The thick solid bars illustrate the expected size of the *Nco*I restriction fragments identified by Southern hybridization with a probe spanning the 3′ flank of the homologous region or the targeting vector. 2 and 3 represent exons 2 and 3 respectively. pA, polyadenylated tail. (**B**) Southern hybridization autoradiography of a series of clones generated by homologous recombination of the targeting vector at one of the perlecan alleles. (**C**) Perlecan expression in a series of homologous recombinant clones. Confluent cultures were preincubated in serum-free 199 medium for 12 h. The serum-free medium was then replaced and the cells were incubated for an additional 24 h, after which the medium was removed and analysed for the presence of perlecan by slot immunoblotting using a perlecan domain III-specific monoclonal antibody and detection by enhanced chemiluminescence. Films were scanned to determine the intensity of the bands and the results were converted into relative absorbance (right-hand panel).

0.2% FCS. A significant attenuation of growth in the perlecan-deficient clones was observed (Figure 2A). Moreover, there was a reduced responsiveness to mitogenic FGF7 (20 ng/ml for 2 days) (Figure 2B). Clone 76, which expressed reduced levels (approx. 50% of wild-type), and clone 6, which showed no perlecan levels, showed intermediate and maximal reduction in growth respectively. Notably, growth experiments in the presence of 10% (v/v) FCS showed a significant growth inhibition for both clones 6 and 9/4 (Figure 2C), indicating that supplementation with serum factors does not restore the growth of these perlecan-deficient cells. Thus perlecan expression is required for optimal *in vitro* growth of these colon carcinoma cells.

Effects of heparin on the mitogenic activity of FGF7 in perlecan-deficient cells

In these experiments we investigated the effects of heparin on the mitogenic activity of FGF7 in wild-type and perlecan-deficient HCT116 cells. Cells were plated at an initial density of 20000/cm², preincubated in serum-free medium for 24 h and then

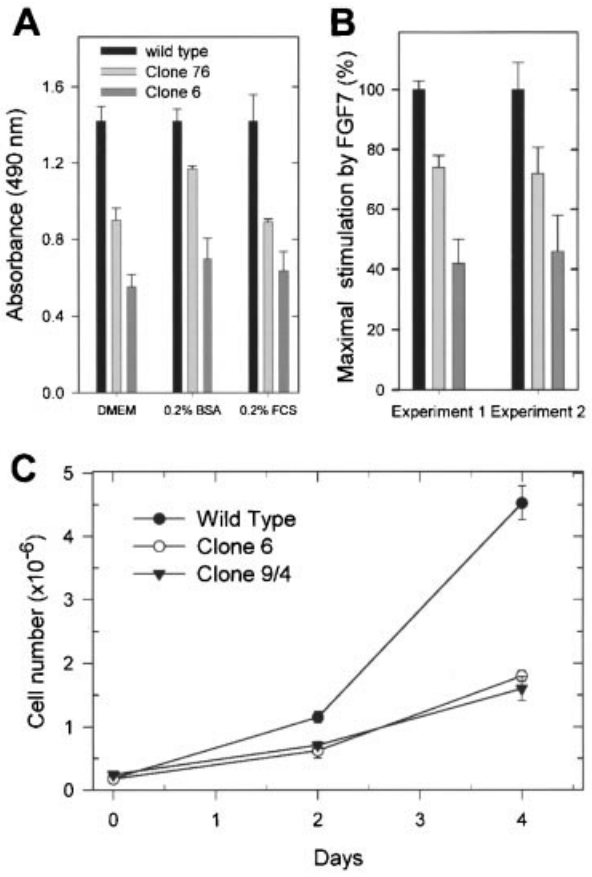

Figure 2 Perlecan is required for the proper growth and mitogenic response to FGF7 in human colon carcinoma cells

(**A**) Growth of various cell types in serum-free medium (DMEM) or in the presence of 0.2% BSA or FCS, as indicated. Growth was monitored following a 2 day incubation using a colorimetric assay (Promega). The values represent the means ± S.D. for triplicate determinations. (**B**) Percentage maximal stimulation by FGF7 (20 ng/ml; for 24 h) in two independent experiments. The values represent the means ± S.D. for triplicate determinations. (**C**) Growth curve of various cells, as designated, in the presence of 10% (v/v) FCS. Medium was changed at days 2 and 4. The values represent the means ± S.D. for triplicate determinations.

incubated in the presence of 20 ng/ml FGF7 and increasing concentrations of heparin (up to 1 μg/ml) for an additional 48 h. The perlecan-deficient cells grew at a significantly slower rate (approx. 80% slower) than wild-type HCT116 cells, both in the presence and absence of exogenous heparin. Heparin had a significant stimulatory effect on wild-type cells at 1 μg/ml (Figure 3), and a lesser, but still significant, effect on clone 76. However, clone 6 was totally unresponsive to the addition of heparin, as was clone 9/4 (results not shown). In separate experiments, we tested the ability of FGF1 to stimulate the growth of perlecan-deficient cells, using a similar protocol, and found no effects (results not shown). Thus perlecan is required for proper FGF7 and FGF1 signalling. The fact that perlecan-deficient cells are refractory to heparin suggests that exogenous heparin cannot substitute for the missing proteoglycan.

Perlecan modulates the cell surface binding of FGF7

Next, we sought to determine whether perlecan deficiency could affect FGF7 interaction with the low- and high-affinity binding sites present at the cell surface. To this end, quiescent confluent

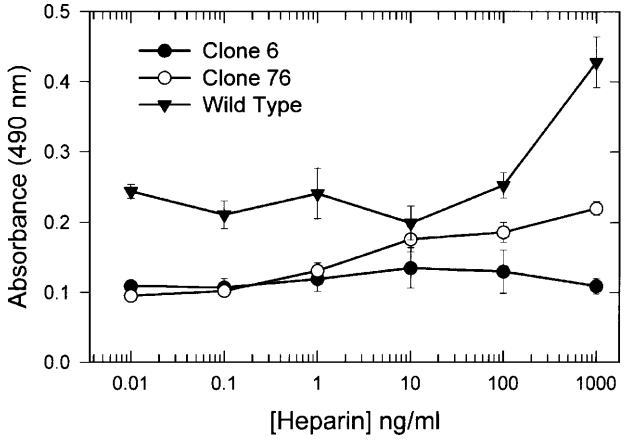

Figure 3 Effects of heparin on the growth of wild-type and perlecan-deficient cells

Approximately 10^4 cells were plated in 199 medium supplemented with 0.4% FCS. After 2 days of serum starvation, the cultures received 20 ng/ml human recombinant FGF7 together with the indicated concentrations of heparin. After 48 h the cell number was assessed using a colorimetric assay. The values represent the means ± S.D. for triplicate determinations.

cells were incubated with increasing concentrations of ^{125}I-FGF7 at 4 °C for 2.5 h. The discrimination between two classes of FGF7 receptor is possible because high salt concentration and pH conditions differentially affect ligand binding [43]. Thus FGF7 bound to the low-affinity receptor, mainly cell surface HS, can be released by short exposure to 1 M NaCl, whereas FGF7 bound to KGFR, the high-affinity FGF7 receptor, is released when cells are washed in an acidic environment [44]. The binding kinetics of ^{125}I-FGF7 revealed that the perlecan-deficient clone 6 displayed greater binding capacity to the low-affinity receptor than either wild-type cells or the parental clone 76 cells (Figure 4, top). In addition, the binding to the high-affinity receptor was also greater in the perlecan-deficient cells (Figure 4, bottom). These results are consistent with the concept that perlecan negatively affects the rate of association and total capacity of FGF7 binding to HCT116 cells, and suggest a role for perlecan in mediating the accessibility of FGF7 to its receptor. However, perlecan is required for full FGF7 biological activity (see below).

Perlecan does not affect the affinity of FGF7 for cell surface HS or KGFR

To analyse further the nature of the cell surface binding of FGF7, wild-type and perlecan-deficient cells were incubated with ^{125}I-FGF7 for 4 h at 4 °C together with increasing concentrations of heparin. The binding interaction was then investigated using BS^3, a homobifunctional, non-permeable cross-linker. In agreement with the experiments shown above, at low concentrations of heparin (1–10 ng/ml), total ^{125}I-FGF7 binding to both low- and high-affinity receptors in perlecan-deficient cells was significantly greater than in either wild-type or clone 76 cells (Figure 5A). However, heparin was equally effective in displacing ^{125}I-FGF7 from all cell types. In fact, an IC_{50} value of approx. 0.1 μg/ml was essentially identical in the three cell types. The specific covalent affinity cross-linking to KGFR was confirmed by SDS/PAGE and autoradiography (Figure 5B). A major complex of approx. 150 kDa was observed in all of the samples, and the displacement by increasing concentrations of heparin followed a pattern similar to that determined by counting

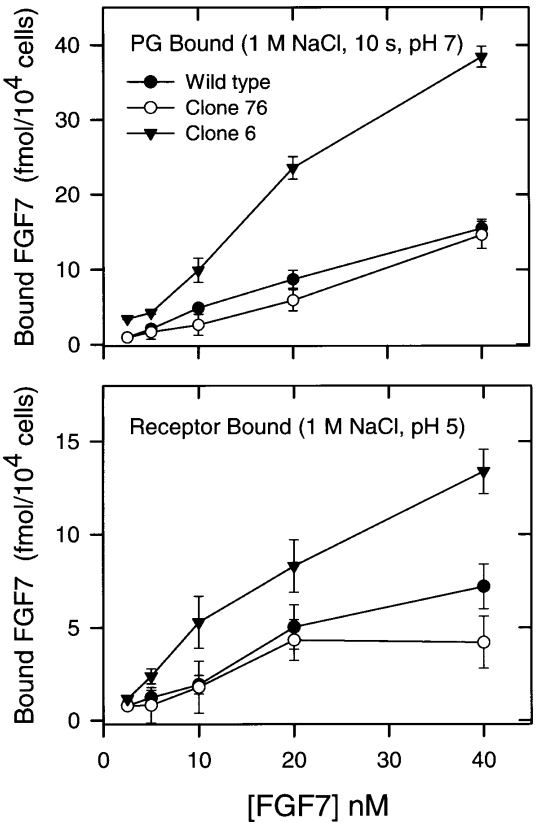

Figure 4 Characterization of the binding site of FGF7 to wild-type and perlecan-deficient cells

Top, proteoglycan (PG) bound ^{125}I-FGF 7 as determined by a rapid wash (approx. 10 s) in 1 M NaCl at pH 7.0. Bottom, receptor-bound ^{125}I-FGF7 as determined by a subsequent extraction with a low pH buffer as indicated. Confluent cells were incubated in 199 medium supplemented with 0.4% FCS for 24 h. On the day of the experiment, the cells were washed with ice-cold binding buffer (DMEM, 15 mM Hepes and 0.05% gelatin, pH 7.4) and incubated at 4 °C for 2.5 h with increasing concentrations of ^{125}I-FGF7. The cells were washed three times with ice-cold binding buffer and the proteoglycan-bound ^{125}I-FGF was removed with a rapid wash (approx. 10 s) using 20 mM Hepes/1 M NaCl (pH 7.0). The residual, receptor-bound radioactivity was then extracted with two consecutive washes in 10 mM sodium acetate/1 M NaCl (pH 5.0). The values represent the means ± S.D. for triplicate determinations.

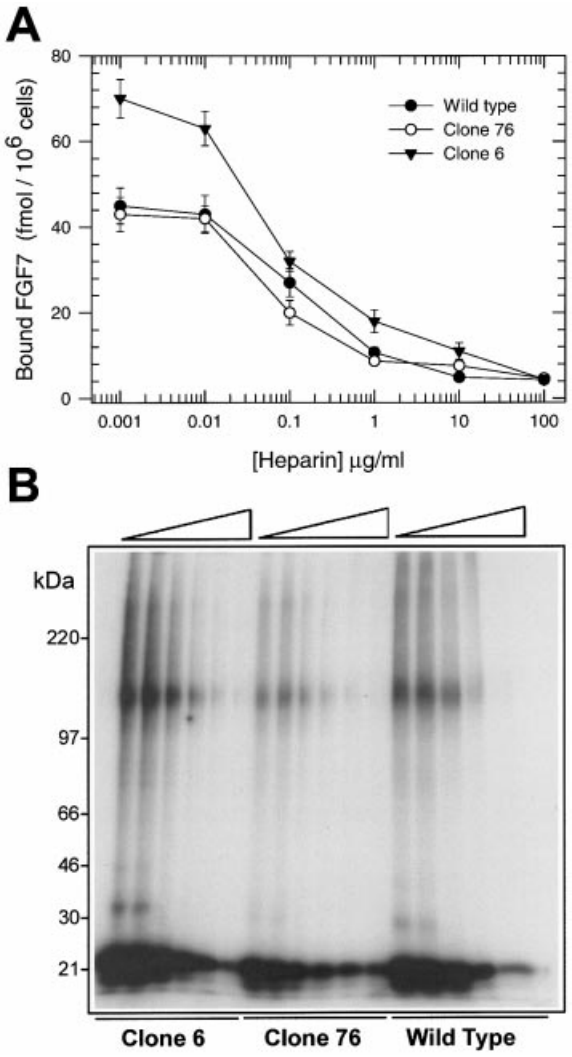

Figure 5 Displacement of surface bound ^{125}I-FGF7 by heparin and cross-linking experiments

(**A**) Displacement curves of cell-surface-bound ^{125}I-FGF7 by increasing concentrations of heparin. Notice that the IC_{50} values are essentially identical in all three cell types (approx. 0.1 μg/ml). Cells were grown to confluence, serum starved for 36 h and then incubated for 4 h at 4 °C with ^{125}I-FGF7 together with increasing concentrations of heparin. The medium was then removed and the cultures incubated at 25 °C in medium containing 1 mM BS^3, a homobifunctional, non-permeable cross-linker. The reaction was quenched 20 min later by the addition of 1 M Tris (pH 7.5) and the proteins were solubilized in Tris buffer containing 0.1% Triton X-100 and a cocktail of protease inhibitors (Complete™; Boehringer Mannheim). Aliquots were counted in a gamma counter. (**B**) Autoradiographic analysis of the affinity cross-linking experiment shown in (**A**). The open triangles depict the increasing concentrations of heparin. The molecular mass markers (in kDa) are shown on the left-hand side. Approximately 50 μg of cells extracts were separated on SDS/PAGE gels [7% (w/v) polyacrylamide] and the cross-linked products were visualized by autoradiography.

of the total cell extracts. The major affinity-labelled complex of approx. 150 kDa is consistent with a ligand:receptor stoichiometry of 1:1 (approx. 125 kDa for KGFR and approx. 21 kDa for FGF7). In agreement with previous studies [45], the majority of the ^{125}I-FGF7 was not cross-linked to its receptor, suggesting that other molecules, probably members of the syndecan/glypican family of cell surface HSPGs, are implicated in surface binding. Collectively, these results indicate that perlecan is not a prerequisite for FGF7 binding to its receptor, but it is required for proper FGF7 functional activity (see below).

Perlecan is required for functional activation of KGFR and downstream signalling

The data reported above suggest the possibility that the perlecan protein core is required for KGFR functionality. First, we determined the basal levels of KGFR in the various cells using immunoblotting with a specific monoclonal antibody directed against the N-terminus of KGFR (anti-Bek monoclonal antibody). The levels of KGFR were essentially undistinguishable among the various cells (Figure 6A). Next, we investigated whether FGF7-mediated signal transduction could be properly triggered in the absence of perlecan. To this end, we incubated wild-type and two perlecan-deficient clones with various concentrations of FGF7 or FGF1, and KGFR and MAPK phosphorylation were assessed by Western immunoblotting (Figure 6B). Short exposure (15 min) of quiescent cells to FGF7 elicited the signalling cascade in wild-type cells, as evidenced by a significant increase in KGFR phosphorylation and, to a greater extent, MAPK phosphorylation (Figure 6B). When tested at

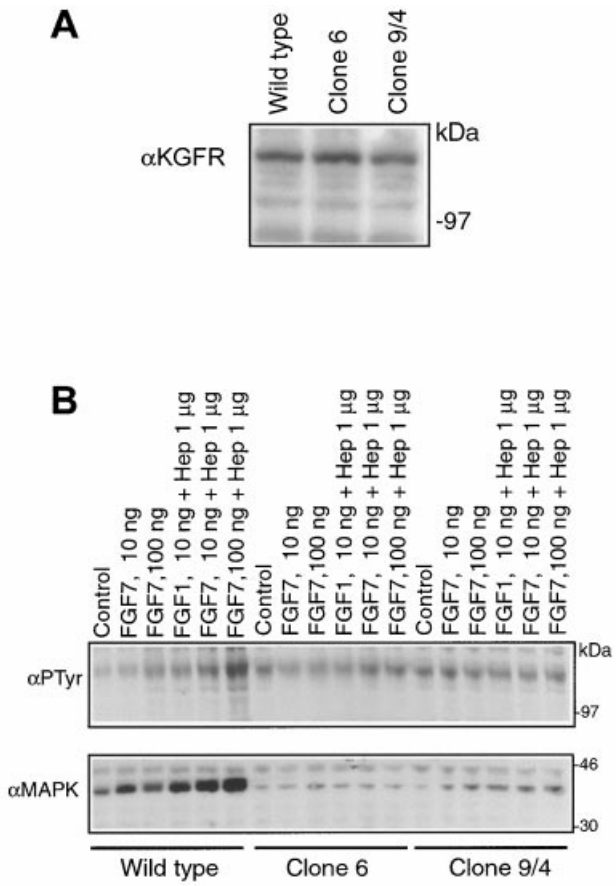

Figure 6 Requirement of perlecan for KGFR and MAPK phosphorylation

(**A**) Basal levels of KGFR as determined by a quantitative immunoblotting assay using approx. 50 μg of cell protein from quiescent cells and a specific monoclonal antibody (anti-Bek monoclonal antibody; C8) directed against the intracellular domain of KGFR/FGFR2. (**B**) Lack of KGFR and MAPK response to recombinant FGFs in perlecan-deficient cells. Quiescent cells were incubated for 15 min with FGF7 or FGF1 with or without heparin (Hep) as indicated. Equal amounts of protein (approx. 50 μg/lane) were separated by SDS/PAGE [7% (w/v) polyacrylamide] and transferred on to nitrocellulose. The filter was cut just below the 97 kDa marker. The top portion of the filter was processed for detection of phosphoproteins by incubation with monoclonal antibodies against phosphotyrosine (αPTyr). The bottom part of the filter was processed for the detection of MAPK phosphorylated products using a mouse monoclonal anti-(MAPK phosphoprotein) antibody (αMAPK) and detection with a rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibody, followed by enhanced chemiluminescence detection.

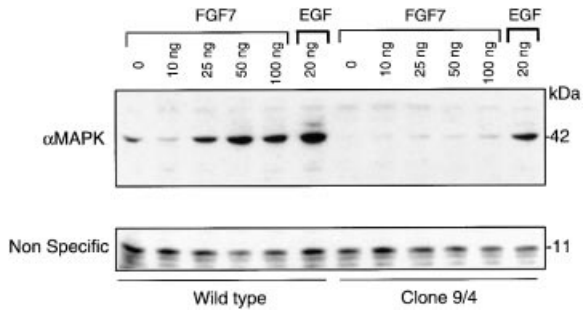

Figure 7 Dose–response stimulation of MAPK phosphorylation induced by FGF7 and EGF in wild-type and perlecan-deficient cells

The top panel shows the level of MAPK phosphorylation, whereas the bottom panel shows an 11 kDa non-specific band for normalization. In these experiments, confluent cells were serum starved for 36 h, and incubated with the indicated concentrations of human recombinant FGF7 or EGF. After a 15 min incubation, the cells were washed three times at 4 °C and extracted in the presence of 0.2% Triton X-100 and a cocktail of protease inhibitors (see the Materials and methods section for details). For Western immunoblotting, approx. 50 μg of proteins were resolved by SDS/PAGE [11% (w/v) polyacrylamide], blotted on to nitrocellulose and MAPK phosphorylated isoforms were detected using a MAPK phosphoprotein-specific monoclonal antibody (New England Biolabs). The immune complexes were reacted with horseradish peroxidase-conjugated anti-mouse IgG followed by chemiluminescence detection using an enhanced chemiluminescence kit (SuperSignal; Pierce). Filters were exposed to X-ray films for 2–10 s (top panel). Representative results for a non-specific 11 kDa protein are shown in the bottom panel (2 min exposure) to show equal loading.

100 ng/ml, FGF7 elicited an even greater response. Notably, FGF7 activity was augmented by the addition of heparin. In contrast, no phosphorylation response could be evoked in perlecan-deficient cells. The response to FGF1 was also investigated in the same set of experiments. Besides FGF7, FGF1 and FGF10 can recognize and activate KGFR/FGFR2b [46–48]. As in the case with FGF7, FGF1-elicited receptor and MAPK phosphorylation were detectable in wild-type cells but not in perlecan-deficient clones (Figure 6B).

Potential problems with the data presented above include the possibility that the targeted clones could have an abnormal response to FGFs or be impaired in receptor tyrosine kinase activity. To address these points, we performed dose–response experiments with increasing concentrations of FGF7 and tested in parallel the response to EGF, which acts through the EGF receptor (EGFR), another well known receptor tyrosine kinase. We found that there was a markedly attenuated response of MAPK phosphorylation at all of the concentrations tested of FGF7 in the perlecan-deficient cells (Figure 7). In contrast, the perlecan-deficient cells responded to the activation of the EGFR, as shown by MAPK phosphorylation (Figure 7). The level of the EGFR was tested by specific anti-EGFR monoclonal antibodies and found to be similar in all of the clones tested (results not shown). These experiments were repeated three times and showed $> 95\%$ inhibition of MAPK phosphorylation in response to FGF7 or FGF1, whereas the response to EGF was only approx. 10–20% lower than wild-type cells.

Our results indicate that perlecan is important for ligand-mediated KGFR phosphorylation. Because heparin alone is ineffective in restoring receptor responsiveness to FGF7 or FGF1, we conclude that lack of the perlecan protein core is responsible for the lack of KGFR phosphorylation in the mutated cells. Moreover, the EGFR signalling pathway is conserved, indicating no inadequacy of global tyrosine kinase activity. Finally, the failure to observe KGFR phosphorylation in the perlecan-null cells indicates that the absence of perlecan causes a defect in signal transduction at the level of the receptor.

Delayed growth of tumour xenografts induced by perlecan-deficient cells

To test whether perlecan deficiency may also play a role *in vivo*, we generated tumour xenografts in immunocompromised (*nu*/*nu*) mice ($n = 10$/group). Notably, the tumours generated by the perlecan-deficient cells showed a significant delay in growth, by 10 days they were one-third of the size of the wild-type tumour xenografts (Figure 8A). By day 24, the wild-type tumour xenografts had reached large dimensions and became ulcerated, whereas the perlecan-deficient tumour xenografts continued a slow pattern of growth. In another set of experiments, we tested

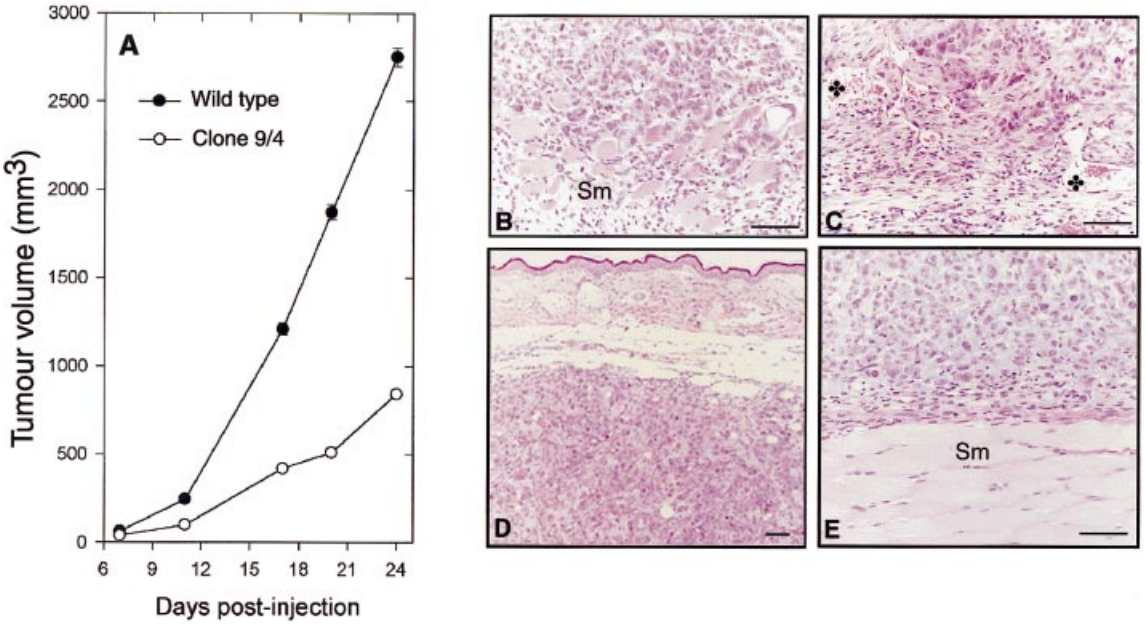

Figure 8 Delayed *in vivo* tumorigenicity in the absence of perlecan

(**A**) Kinetics of tumour growth induced by the subcutaneous injection of wild-type or perlecan-deficient colon carcinoma cells. Tumour xenografts were generated by injecting approx. 10^6 cells into the mid-dorsum of immunocompromised (*nu/nu*) mice ($n = 10$/group). The values represent the means $\pm$ S.D. Two independent experiments were performed with similar results. (**B** and **C**) Morphology of wild-type tumour xenografts. Notice the infiltration of skeletal muscle (**B**; Sm) and the prominent neovascularization (**C**; asterisks). (**D** and **E**) Morphology of perlecan-deficient tumour xenografts. Notice the sharp borders of growth (**D**) and the lack of infiltration of the subcutaneous skeletal muscle (**E**). Scale bars = 50 μm.

wild-type cells and the heterozygous clone 76 ($n = 9$/group) and found a similar pattern of *in vivo* growth (results not shown). All the wild type-generated tumour xenografts revealed extensive invasion of the deep fascia and subcutaneous skeletal muscles (Figure 8B), together with abundant angiogenesis (Figure 8C). In contrast, the perlecan-deficient tumour xenografts showed very sharp margins and no infiltration of the deeper, soft tissues (Figures 8D and 8E). However, since the animals were killed simultaneously at the end of the experiment, we do not know the development of the perlecan-deficient xenografts. Collectively, these data substantiate the *in vitro* experiments and further indicate that perlecan is important for *in vivo* tumorigenesis and angiogenesis.

DISCUSSION

One of the most direct approaches for elucidating the role of a specific gene product in a complex *in vivo* system is to develop mutants that no longer express the gene. Through somatic gene knockout and clonal selection, we have identified perlecan as a necessary component for the FGF7-mediated activation of KGFR, both in terms of its initial activation and long-term effects on growth and tumorigenicity. This has allowed us to address directly the question of the functional role of perlecan in FGF7-mediated signal transduction. Compared with the parental wild-type cells, binding of FGF7 to the mutated HCT116 cells was paradoxically higher, although the affinity was similar. At the same time the binding to the low- and high-affinity cell surface sites was increased. In a previous study we found that antisense blocking of perlecan gene expression did not cause a significant change in high-affinity binding sites for FGF7 in the same cells [32]. These apparently contradictory results might be in part explained by the fact that in the antisense-treated cells, 5–10 % of the endogenous perlecan was still present, whereas in the present study perlecan expression was completely abolished. Moreover, we cannot exclude the possibility that additional cell surface molecules might be involved in mediating FGF7 binding. Future studies need to be performed to address these points.

Our results further show that in spite of the increased cell-surface occupancy, both basal and FGF7-induced phosphorylation of the KGFR were markedly attenuated in the absence of the perlecan protein core. Lack of FGF7-mediated activation in perlecan-deficient cells was further confirmed by the absence of MAPK phosphorylation. Like FGF7, FGF1, another known ligand for KGFR, was also unable to elicit tyrosine kinase receptor and MAPK phosphorylation, consistent with a direct involvement of perlecan in the initial steps of KGFR activation. Concurrent activation of the EGFR kinase indicates that perlecan is not directly involved in this signalling pathway. Notably, the perlecan-deficient cells grew more slowly *in vitro* and exhibited a delayed growth *in vivo* with decreased neovascularization, indicating that this gene product is important for growth and tumour angiogenesis.

Studies performed with solubilized KGFR ectodomain and FGF7 have shown that HS chains can facilitate FGF7 binding to its receptor [19,49]. The relevance of these studies is based on the concept that the formation of the binding complex is a prerequisite for receptor activation through phosphorylation of its intracellular domain [50,51]. In the absence of heparin/HS, the concentration of FGF7 required for signal transduction exceeds what is expected under normal physiological conditions. Given the low affinity of FGF7 for its receptor, it is unlikely that the first step in receptor interaction *in vivo* is the binding of the growth factor to KGFR [19]. The favoured scenario is rather an initial interaction of either the receptor or FGF7 with HS prior to growth factor binding and receptor activation. The kinetics of

formation of FGF7–KGFR–heparin ternary complexes in solution have shown that a single FGF7 molecule binds sequentially to two KGFR ectodomains and that heparin/HS is necessary for the formation of the initial bimolecular complex [19]. The binding site for heparin is located in the second Ig module of KGFR, and because this modular domain is present in all of the FGFRs, it is likely that the flanking structural domains may modify the KGFR affinity for the different ligands and, thus, determine its specificity for FGF7 [49]. The heparin-binding site on FGF7 resides at the N-terminus of FGF7 and is spatially juxtaposed to the binding site for the KGFR located at the C-terminus [52]. According to this structural model, the binding of heparin to FGF7 would cause a folding of the molecule allowing KFGR recognition. However, depending on the cell context, HS might not be required for binding and internalization of other FGFs, such as FGF2 [53], and FGF2 oligomers can be formed in the absence of HS [54]. Moreover, FGF2–FGFR crystals can form in the absence of heparin [55]. A minimal functional complex is supported by two sets of independent experiments in which cross-linked FGF2/heparin monomers activate FGFR in HS-deficient cells [56] and in which FGF7 forms heparin-dependent 2:1 complexes with soluble KGFR [19]. Co-crystallographic analyses have shown that heparin interacts via its non-reducing ends with both FGF2 and FGFR, and promotes the formation of a 1:1:1 ternary complex, which in turn attracts another ternary complex, thereby forming a hexameric structure [57]. This is in partial contrast with a pentameric structural model in which a complex is assembled around a central heparin molecule linking two FGF1 ligands into a dimer that bridges between two receptor chains [58]. In both models, however, heparin plays a key role by stabilizing both FGFR–FGFR and FGF–FGFR interactions. Cell surface perlecan could play a dual role *in vivo* by binding FGF7 via either its HS chains or protein core domains III and V [33]. Because the HS chains located in N-terminal domain I can be quite long, reaching 100 nm in length, it is conceivable that FGF7 might interact simultaneously with both HS chains and the perlecan protein core.

Ectopic expression of KGFR in BaF3 cells, that lack both HSPGs and FGFRs, leads to binding of FGF7 to the receptor, a process enhanced by low concentrations of heparin [59]. However, at higher concentrations ($> 10\ \mu$g/ml), heparin is inhibitory. This biphasic behaviour of heparin has also been observed in CHO/745 cells [45], another cell line that does not express HSPGs. When stably transfected with KGFR, these cells become responsive to FGF7. Heparin potently stimulates the mitogenic response in a dose-dependent fashion up to 1 μg/ml, but becomes inhibitory at higher concentrations. Notably, in these cells, heparin inhibits the mitogenic response to FGF7 even at the lowest concentrations tested. These data suggest that other molecules present at the cell surface might be involved in modulating FGF7 binding and signalling, and raise questions about the specificity of the effects brought about by heparin.

Glypican abrogates the stimulatory effect of heparin in cells ectopically expressing KGFR [59,60]. The mechanism of this effect does not involve a direct interaction of the HSPG with the receptor. Glypican may rather act as a competitive inhibitor of stimulatory HSPGs for FGF7. A number of studies indicate that binding competition for FGFs between HSPGs and their receptors may be an important determinant for biological activity and specificity of action. For example, syndecans can have inhibitory effects on heparin-dependent FGF2 receptor binding [15]. However, when tested in the absence of heparin for their ability to enhance FGF2 binding to FGFR1, syndecans are stimulatory [61]. Thus heparin might compete with the HS chains of syndecans for binding to either the growth factor or the tyrosine kinase receptor, thereby identifying the HS chains as the biological entity responsible for growth factor binding and receptor activation. This has also been demonstrated for glypican, inasmuch as the HS chains obtained by digestion of the protein core can stimulate FGF2 binding, whereas digestion with heparinase abolishes glypican activity [60]. In addition, both glypican and heparin inhibit FGF7, but not FGF1, binding to KGFR, further suggesting that the HS chains are involved in modulating ligand receptor specificity. Our results, showing that FGF7 binding to KGFR is inhibited by heparin in both wild-type and perlecan-deficient cells, are consistent with the concept that perlecan limits FGF7 binding to the cells surface receptors, since the cells specifically lacking the proteoglycan display higher binding capacity than the wild-type cells. However, the kinetics of FGF7 displacement by heparin are essentially the same (IC_{50} values of approx. 0.1 μg/ml). This suggests that the perlecan protein core may regulate the specific targeting of the ligand to its own receptors, since perlecan deficiency significantly attenuates KGFR activation.

In previous studies we showed that FGF7 mitogenic activity is largely dependent on perlecan availability [32], and that domain III of perlecan, the region homologous to the short arm of laminin α1 chain, harbours a high-affinity docking site for FGF7 [33]. The present study extends these observations to document that perlecan is required for FGF7-mediated KGFR phosphorylation through a mechanism involving the proteoglycan protein core. The evidence that the heparin-sensitive FGF7 binding to KGFR takes place independently of perlecan, and that heparin cannot restore FGF7 functionality in perlecan-deficient cells, argues against a significant role of perlecan HS chains in KGFR phosphorylation and signal transduction. Rather, the protein core appears to be the molecular determinant involved in receptor tyrosine kinase activation.

Besides the presence of high-affinity binding sites for FGF7, there is evidence that the perlecan protein core can bind other growth factors and an increasing number of extracellular matrix proteins [23]. Given this extraordinary binding heterogeneity, perlecan may be regarded as a scaffold for interacting molecules in the extracellular matrix and the cell surface. The multi-domain protein core may facilitate interactions between otherwise distant molecules allowing local assembly and complex formation. This biological property would fit well with the evidence that this proteoglycan is critically involved at all steps of development and tissue remodelling [21] that require temporal and spatial co-ordination of multiple factors. In the case of cancer, over-expression of perlecan would provide a clear clonal advantage over populations of tumour cells with lower expression.

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