Published in final edited form as:

Arch Biochem Biophys. 2007 December 15; 468(2): 244–251. doi:10.1016/j.abb.2007.10.006.

THE CORE PROTEIN OF GROWTH PLATE PERLECAN BINDS FGF-18 AND ALTERS ITS MITOGENIC EFFECT ON CHONDROCYTES

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Abstract

Fibroblast growth factor-18 (FGF-18) has been shown to regulate the growth plate chondrocyte proliferation, hypertrophy and cartilage vascularization necessary for endochondral ossification. The heparan sulfate proteoglycan perlecan is also critical for growth plate chondrocyte proliferation. FGF-18 null mice exhibit a skeletal dwarfism similar to that of perlecan null mice. Growth plate perlecan contains chondroitin sulfate (CS) and heparan sulfate (HS) chains and FGF-18 is known to bind to heparin and to heparan sulfate from some sources. We used cationic filtration and immunoprecipitation assays to investigate the binding of FGF-18 to perlecan purified from the growth plate and to recombinant perlecan domains expressed in COS-7 cells. FGF-18 bound to perlecan with a K_d of 145 nM. Near saturation, ~103 molecules of FGF-18 bound per molecule of perlecan. At the lower concentrations used, FGF-18 bound with a K_d of 27.8 nM. This binding was not significantly altered by chondroitinase nor heparitinase digestion of perlecan, but was substantially and significantly reduced by reduction and alkylation of the perlecan core protein. This indicates that the perlecan core protein (and not the CS nor HS chains) is involved in FGF-18 binding. FGF-18 bound equally to full length perlecan purified from the growth plate and to recombinant domains I-III and III of perlecan. These data indicate that low affinity binding sites for FGF-18 are present in cysteine-rich regions of domain III of perlecan. FGF-18 stimulated ³H-thymidine incorporation in growth plate chondrocyte cultures derived from the lower and upper proliferating zones by 9- and 14-fold, respectively. The addition of perlecan reversed this increased incorporation in the lower proliferating chondrocytes by 74% and in the upper proliferating cells by 37%. These results suggest that perlecan can bind FGF-18 and alter the mitogenic effect of FGF-18 on growth plate chondrocytes.

Introduction

Perlecan is a large (~600 kDa) heparan sulfate-containing proteoglycan present in all mammalian basement membranes [1,2], in cartilage [3,4] and in the growth plate [5]. Perlecan is essential for long bone growth. The absence of perlecan in mice results in defective endochondral ossification (the process by which long bones grow) during embryonic

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development [6,7]; the chondrocytes in the growth plate of perlecan null mice exhibit decreased proliferation and matrix deposition, which leads to severe fetal dwarfism. Silverman-Handmaker dyssegmental dysplasia (DDSH), which results from a functional null mutation of the perlecan gene [8,9] is an analogous disorder in humans. Schwartz-Jampel syndrome (SJS) in humans is thought to result from production of truncated (or reduced levels of full-length) perlecan [10], with patients showing dwarfism and skeletal abnormalities that mirror those of DDSH patients but are less severe.

Fibroblast growth factor receptor-3 (FGFR-3) is a cell surface receptor expressed in developing growth plate cartilage [11]. Gain of function mutations in the human FGFR-3 gene cause dwarfisms such as achondroplasia [12], thanatophoric dysplasia [13], and hypochondroplasia [14]. Activating mutations in murine FGFR-3 also cause dwarfism [15–17]. When FGFR-3 expression in the growth plate of mice is eliminated, there is increased chondrocyte proliferation and hypertrophy resulting in overgrowth of long bones [18,19]. This indicates that FGFR-3 is a negative regulator of chondrocyte proliferation.

Fibroblast growth factors (FGF's) are the endogenous ligands for FGFR's. The FGF-2 knockout mouse has no severe defect in bone length [20] whereas over-expression of FGF-2 in the FGF-2 transgenic mouse results in short limbs, indicating that FGF-2 can act through FGFR-3 [21,22]. Both the FGF-9 transgenic mouse and the FGF-18 knockout mouse show severe abnormalities in long bone growth [23–25], but it is the FGF-18 null mouse that exhibits a growth plate phenotype similar to that of the FGFR-3 null mouse, in the increase (although transient) in chondrocyte proliferation and in enlarged growth plates [24,25]. FGF-18 has a three-tiered importance in long bone growth, affecting chondrogenesis by signaling through FGFR-3 [26], osteogenesis by signaling through FGFR-1 and/or FGFR-2 [24,25], and growth plate vascularization by regulating vascular endothelial growth factor (VEGF) [27]. FGF-18 is made by the developing perichondrium, the region of connective tissue that surrounds the developing growth plate [24,25].

All FGF's bind to heparin [28] and heparin has been shown to enhance the binding of FGF's to FGFR's [29]. Heparan sulfate (HS), a structural analogue of heparin, is present on growth plate perlecan at both ends (domains I and V) of the core protein [30,31]. Perlecan binds FGF-2 via these HS chains and can mediate the delivery of FGF-2 to FGFR-1 and FGFR-3 [32]. FGF-18 is known to bind preferentially to 2-O-sulfated HS [28] and the HS on growth plate perlecan is 2-O- and 6-O-sulfated [33]. Consequently, in addition to binding FGF-2, the HS on perlecan may also bind FGF-18. In this study, we test FGF-18 as a potential binding partner of growth plate perlecan and as a mitogen for chondrocytes isolated from the developing rib growth plate.

Materials and methods

Sources of perlecan DNA, perlecan protein, growth factors and antibodies

cDNA constructs encoding for perlecan domain I-III and for domain III were obtained from previous work [34,35]. Large-scale plasmid preps were prepared using the Endo-free Plasmid Maxi Kit (QIAGEN) and constructs were verified using restriction endonucleases. Recombinant human fibroblast growth factor-18 (FGF-18) was purchased from PeproTech. Purified bovine growth plate perlecan [5] and anti mouse perlecan antibody Ab378 [33] were prepared as previously described.

Transfection of perlecan into COS-7 cells

High glucose (4.5 g/L) DMEM (Fisher Scientific) was supplemented to 10% fetal bovine serum, 1% antibiotics (penicillin-streptomycin) and 2% glutamine and used as our growth

medium to culture COS-7 cells in 100 mm cell-culture dishes (Fisher Scientific). Lipofectamine 2000 (L2000, Invitrogen) was used to transfect confluent COS-7 cells according to the manufacturer's directions. Briefly, the cells were rinsed with serum- and antibiotic-free growth medium (our "transfection medium"). For the constructs Alt1 (domains I-III) and Alt2 (domains I-III made without heparan sulfate), cells were transfected with 12 µg of DNA using 45 µl of L2000 in 15 ml of transfection medium. For the domain III construct, cells were transfected with 18 µg of DNA using 45 µl of L2000. The cells were cultured at 37°C/5% CO₂ for 5h, then changed to growth medium for another 16–18h. The transfected cells were then further cultured in 15 ml of fresh transfection medium and harvested after 48h.

Purifying Recombinant Products

The 48h media from transfected COS-7 cells were reconstituted to 4 M guanidine HCl by the addition of 0.5 g of guanidine HCl/ml of media. The media were then spin concentrated to less than 2 ml and fractionated on 4 M guanidine HCl-equilibrated Superose 6 columns (GE Healthcare). Forty 3 ml fractions (1.2 column volumes) were collected, then 1 µl of each fraction was dotted onto nitrocellulose membrane (Bio-Rad) and immunoblotted for perlecan using Ab378. Perlecan-positive fractions were dialyzed against distilled water and 18 µl aliquots of the fractions were run on SDS-PAGE. Fractions from the Alt1 column run were digested with 10 mU of protease-free chondroitinase ABC and 5 mU each of heparitinases I and II (Associates of Cape Cod) before SDS-PAGE, as described below. The DIII and Alt2 fractions were not digested since these recombinant products have no GAGs attached. Fractions determined by Western blot to contain the recombinant product but no endogenous COS-7 cell perlecan were pooled and the amount of perlecan estimated by direct comparisons with known amounts of EHS perlecan in the dimethylmethylene blue (DMMB) assay [36] and in Western blots.

Enzyme Digestion of Perlecan GAGs

Where indicated in the results, perlecan was either sham digested, digested with 10 mU of chondroitinase ABC or digested with 5 mU each of heparitinases I and II before being used in the CAF or the IP assays. Digestions were done in 100 µl of digestion buffer (20 mM Tris HCl, 5 mM calcium chloride and 0.2 mg/ml protease-free BSA, pH 7.4) at 37°C for 3h. The action of the enzymes on perlecan was monitored by SDS-PAGE. Digestions resulted in a shift in the migration of perlecan to a slightly lower molecular weight, indicating removal of the GAG chains.

Radiolabelling of growth factor

Sodium ¹²⁵Iodide (0.5 mCi, PerkinElmer) activated in IODO-GEN-coated tubes (Pierce) was used to radiolabel 5 µg of FGF-18 using Pierce's supplied protocol (Chizzonite indirect method) as previously described for FGF-2 [32]. The iodinated FGF-18 was applied to a 0.3 ml column of Heparin Sepharose 6 Fast Flow beads (GE Healthcare) and the bound FGF-18 eluted with 2 M NaCl. The purified FGF-18 was dialyzed against PBS and the specific activity of the ¹²⁵I-FGF-18 determined as before [32].

Cationic Filtration Assay

The binding of ¹²⁵I-FGF-18 to perlecan was determined using a cationic filtration (CAF) assay as previously described [32,37]. The binding buffer was 0.05 M Tris HCl, 0.15 M NaCl and 2 mg/ml protease-free BSA, pH 8.0. Briefly, iodinated FGF-18 was incubated with or without perlecan in binding buffer at room temperature for 1h in a final incubation volume of 200 µl. The mixture was then filtered through cationic Zeta-Probe membrane (Bio-Rad) by vacuum filtration using a Minifold-I 96-well apparatus (Schleicher and Schuell, Keene, NH, USA). The wells were rinsed to remove unbound ¹²⁵I-FGF-18, the membrane was dried and the wells

excised and counted in a gamma counter. For the initial dose-response CAF assay, a saturation binding curve and a Scatchard plot of the data were generated using the SigmaPlot Regression Wizard® (SigmaPlot 8.0).

Immunoprecipitation Assay

The binding of FGF-18 to perlecan was studied using an immunoprecipitation (IP) assay as previously described [32,38]. Purified recombinant perlecan was reconstituted to 0.2 ml in IP buffer (1% Triton X-100, 20 mM Tris HCl, 0.15 M NaCl, pH 7.4). ¹²⁵I-FGF-18 was added and the samples were incubated at room temperature for 1h with mixing. Five microliters of either anti- mouse perlecan antibody Ab378 (or 5 µl of pre-immune rabbit serum as control) were added and the samples incubated at 4°C for 2h. After addition of 20 µl of Protein G sepharose beads (GE Healthcare) for 2h at 4°C, the samples were centrifuged for 5 min at 2500×g, the beads washed three times with IP buffer and the radioactivity in the pellets or in 2 M eluates measured in a gamma counter. Perlecan was also either sham-digested or digested with protease-free chondroitinase ABC and heparatinases I and II before being used in the IP assay.

Reduction and Alkylation of Perlecan

Purified growth plate perlecan was adjusted to 8 M Urea/0.2 M Tris HCl (pH 8.5), DTT (Invitrogen) was added at 50 mM and the reaction was incubated for 45 min at 55°C. Iodoacetamide (Pierce) was then added at 100 mM for 20 min at room temperature in the dark. The perlecan sample was then dialyzed against distilled water before being used in the CAF assay.

Chondrocyte isolation and culture

Chondrocytes were isolated from the lower proliferating, upper proliferating, intermediate and resting zones of growth plates from second trimester fetal bovine ribs as previously described [39]. Chondrocyte cultures were plated in DMEM at 10⁶ cells/well in a Costar 6-well plate (Fisher) and allowed to attach for 4 hrs. They were then cultured in DMEM ± 25 ng/ml FGF-18 ± 50 ng GAG/ml of purified growth plate perlecan for 18 hrs. The medium was then supplemented with 5 µCi ³H-thymidine/ml and the cells cultured for an additional 6 hrs. The medium was removed and the cell layer was rinsed with PBS, frozen, thawed and then solubilized in lysis buffer containing fluorescent DNA-binding dye (CyQUANT, from Invitrogen). An aliquot of the lysate was taken to determine DNA content (according to the manufacturer's instructions) and using calf thymus DNA as a standard. The ³H-thymidine incorporated into DNA in the lysate was measured by precipitating the DNA in an aliquot of the cell lysate with 10% TCA overnight at 4°C with 100 µg BSA as a carrier. The precipitate was then collected by vacuum filtration on glass fiber filters (Fisher). The filters were washed three times with 5% TCA and the ³H-counts remaining were measured in a scintillation counter. The incorporation was expressed per ng DNA.

Statistics

All data are expressed as the mean of four replicates ± standard error of the mean. Statistical comparisons were made using Student's t-test, where applicable. P<0.05 was considered significant.

Results

Binding of FGF-18 to full-length perlecan in the CAF assay

¹²⁵I-FGF-18 (33 – 2385 ng/ml; 0.75 – 109 nM) was incubated with or without 8 ng/ml of perlecan (in DMMB GAG content, therefore 40 ng of total perlecan/ml reaction). The samples

were filtered over a cationic membrane and radioligand binding to the membrane was determined using a gamma counter. Counts from incubations without perlecan were subtracted from those with perlecan at equivalent FGF-18 concentrations to determine the specific binding. The nanomolar amounts of FGF-18 corresponding to the bound counts were calculated from the specific activity of the labeled FGF-18 and are shown (Fig. 1A). The data show that the binding of FGF-18 to perlecan was saturable above 84.5 nM FGF-18 added, with a dissociation constant (K_d) of 145 nM as determined from SigmaPlot Scatchard analysis. Figure 1B shows in more detail the low-concentration area outlined by the dashed rectangle in Fig. 1A. The K_d for these five lowest FGF-18 concentrations used is 27.8 nM.

Effect of Enzyme Treatment of Perlecan on FGF-18 Binding in the IP assay

Growth plate perlecan contains both CS and HS chains [5]. We used the IP assay to determine if ^{125}I -FGF-18 could bind to perlecan without intact GAG chains present. Purified perlecan (30 ng GAG/ml) was either sham digested (sham, Fig. 2) or digested with chondroitinase (C) or heparitinases I and II (H) or a mixture of both (C+H). The perlecan was then incubated with ^{125}I -FGF-18 (40 ng/ml) and serum (pre-immune or anti-perlecan), captured with Protein G beads and the FGF-18 binding determined. There was significantly more binding of ^{125}I -FGF-18 to the beads with anti-perlecan serum than with pre-immune serum. There was no significant difference, however, whether the perlecan used had its GAG chains intact (sham treatment) or had only one type of GAG (C or H treatment) or had no intact GAGs (C+H treatment).

Effect of Reduction and Alkylation of Perlecan on ^{125}I -FGF-18 Binding in the CAF assay

To determine if disulfide-bonded core protein structure is involved in FGF-18:perlecan binding, we used 8 ng of FGF-18 in a CAF assay with 8 ng GAG of either perlecan that was denatured with 8 M urea alone or perlecan that had been denatured with urea, reduced with 50 mM DTT and alkylated with 100 mM Iodoacetamide (Figure 3). FGF-18 bound well to the denatured, dialyzed perlecan (8M Urea bar in Figure). However, the binding of FGF-18 to perlecan was reduced by more than 60%, almost to background binding (– bar), when the core protein structure was altered by reduction and alkylation (Urea/DTT/Iodo bar).

Binding of ^{125}I -FGF-18 to full length perlecan and to Alt1 in the CAF assay

For Fig. 4, the binding of ^{125}I -FGF-18 to full-length perlecan was compared to that of AltI, a recombinant product containing only the N-terminal domains I-III of perlecan [34]. At concentrations well below saturation (as determined in Fig. 1), ^{125}I -FGF-18 (20 ng/ml in Fig. 4A, 160 ng/ml in Fig. 4B) was incubated with full-length perlecan or recombinant Alt1 (10 ng GAG/ml). Both perlecan products had similar binding capacity (~50%) for ^{125}I -FGF-18 at both FGF-18 concentrations used.

Binding of ^{125}I -FGF-18 to perlecan domains I-III and to domain III in the IP assay

The domain in the N-terminal half of perlecan that binds FGF-18 was determined in the IP assay by incubating 40 ng/ml of ^{125}I -FGF-18 without perlecan, or with 150 ng/ml of a recombinant product for domain III of perlecan [35] or with Alt1 or with Alt2, a recombinant product of domains I-III made without GAGs [34]. FGF-18 bound equally to domain III as it bound to the domain I-III products (Fig. 5).

Effects of FGF-18 and perlecan on ^3H -thymidine incorporation in growth plate chondrocytes

Chondrocytes were isolated from the lower proliferating, upper proliferating, intermediate and resting zones of the fetal bovine rib growth plates. They were cultured with or without FGF-18 and with or without growth plate perlecan and the incorporation of ^3H -thymidine measured to determine the proliferative activity of the cells. Addition of 25 ng FGF-18/ml (Fig. 6, 25F in

legend) increased ^3H -thymidine incorporation by less than 1-fold in cells from the intermediate (I) and resting (R) zones of the growth plate (Fig. 6B) but did increase incorporation 9-fold and 14-fold in chondrocytes from the lower proliferating (LP, Fig. 6A), and upper proliferating (UP) zones, respectively. Addition of 50 ng perlecan GAG/ml (50P in legend) to chondrocytes cultured in FGF-18 reversed the FGF-18 stimulation by 74% in LP chondrocytes and by 37% in UP chondrocytes. Addition of perlecan alone to the cells from the proliferating zones caused no significant change in ^3H -thymidine incorporation.

Discussion

In this study we used a cationic filtration (CAF) assay and an immunoprecipitation (IP) assay to identify FGF-18 as a novel binding partner of growth plate perlecan. FGF-18 bound to native perlecan purified from the developing growth plate (Fig. 1). The interaction was stronger at low concentrations (0.75 – 24.15 nM FGF-18 added, Fig. 1B) but did not saturate until after 84.5 nM (Fig. 1A). Removal of the chondroitin sulfate (CS) and heparan sulfate (HS) chains of perlecan did not significantly reduce FGF-18 binding (Fig. 2). Thus, even though we used heparin sepharose affinity chromatography to purify the iodinated FGF-18, it did not bind to the HS chains on perlecan. Studies have shown that not all HS has the sequence or sulfation pattern necessary to bind to FGF-18 [38]. However, when the perlecan core protein is reduced and alkylated (Fig. 3), FGF-18 binding to perlecan approaches background binding. These data indicate that no other growth plate molecules are needed to mediate the interaction between perlecan and FGF-18 and that the binding of FGF-18 to perlecan is dependent upon cysteine-mediated folding of the perlecan core protein.

FGF-18 also bound equally well to the recombinant products Alt1, Alt2 and DIII of perlecan made in COS-7 cells (Fig. 4 and Fig. 5). Purified full-length perlecan from the growth plate contains 25% of its total GAGs as HS and 75% as CS, Alt1 contains 60% HS and 40% CS, and Alt2 does not contain GAGs, yet they all bind FGF-18 comparably. This suggests that neither the intact GAGs on perlecan nor the GAG stubs after glycosidase digestion influence FGF-18 binding. The Alt1 product is the N-terminal third of domain I containing the serine GAG attachment sites plus all of domains II and III [34]. The Alt2 product is made without GAGs since the serines in domain I that are GAG attachment sites were mutated to threonines [34]. DIII also bound equally well to FGF-18 as Alt1 and Alt2 did, which pinpoints the binding site of FGF-18 to domain III of perlecan (Fig. 5).

The GAG on perlecan constitutes 20% of its molecular mass [5,31,34]. Therefore, the 1.6 ng/tube (8 ng/ml) of perlecan GAG used in our initial dose-response CAF assay (Fig. 1) corresponds to 8 ng of perlecan. The molecular masses of perlecan and FGF-18 are ~600 kDa [32] and 22 kDa (Peprotech product data sheet) respectively. As determined from our saturation binding curve shown in Figure 1A, near saturation (i.e. at 84.5 nM FGF-18 added) our calculations yield a stoichiometry of ~103 FGF-18 molecules bound per molecule of perlecan. This suggests that growth plate perlecan is capable of serving as a reservoir for large amounts of FGF-18, a function previously shown for perlecan with other growth factors [32,40,41]. Scatchard analysis of the complete binding data using SigmaPlot showed a K_d of 145 nM for the interaction between FGF-18 and perlecan (Figure 1A, inset). The affinity constant for the five lowest concentrations used was 27.8 nM (Fig. 1B, inset), which shows increased affinity relative to the higher concentrations used but, still, relatively a low affinity interaction.

Domain III has previously been shown to bind to a number of proteins including integrins, FGF-7, FGF-BP (FGF binding protein), PDGF (platelet derived growth factor) and WARP (von Willebrand factor A domain-related protein, which is expressed in chondrocytes and affects ECM structure) [35,41–44]. We show here that domain III binds FGF-18. Domain III has a calculated molecular mass of 120–135 kDa [30], accounting for ~25% of the mass of

total core protein, and is organized into three identical subdomains that show extensive tertiary structure. Each of the three subdomains consists of a 28–41 amino acid cysteine-rich repeat followed by a 192–199 amino acid cysteine-free globular domain followed by three additional cysteine-rich repeats (totaling 147–168 amino acids) [30]. The repetitive structure of domain III (which would provide multiple binding sites) and the tendency of fibroblast growth factors to multimerize [45] may explain the 103:1 stoichiometry of the FGF-18:perlecan interaction; the binding of FGF-18 to perlecan could enhance FGF-18 multimerization. Since reduction and alkylation would disrupt cysteine-cysteine bonding, it is likely that FGF-18 is binding to the cysteine-rich regions of domain III. The disulfide-bonded cysteine-rich repeats of domain III are homologous to epidermal growth factor (EGF) and confer a resistance to proteolysis [35]. We can speculate, then, that a growth factor (such as FGF-18) bound to this stable domain III might be more effectively protected in the extracellular matrix.

The results of our study showed that FGF-18 stimulated ³H-thymidine incorporation in chondrocytes from the proliferating zones far more than in chondrocytes from the intermediate or resting zones. This suggests that chondrocytes acquire the ability to respond to FGF-18 as they differentiate in the growth plate. Analysis of the growth plates of FGF-18 null mice indicates that FGF-18 would act to stimulate proliferation of growth plate chondrocytes at early stages of development [27] and then to inhibit proliferation in later embryonic/postnatal development [24]. The chondrocytes in our study are from second trimester calves; they are earlier in development than cells from the late embryonic/postnatal stage and, thus, FGF-18 significantly stimulated ³H-thymidine incorporation in chondrocytes from these developing growth plates. We also found that perlecan significantly reversed the FGF-18-mediated stimulation of ³H-thymidine incorporation in chondrocytes from the lower proliferating and upper proliferating zones. This suggests that perlecan can modulate the effect of FGF-18 on growth plate chondrocyte proliferation.

FGF-2 is another low affinity binding partner of perlecan but binds exclusively via perlecan's HS chains [32,38,46]. The HS attachment sites in domain I of perlecan have been deleted in the mouse model *hspg2^{Δ3/Δ3}* [47], but the perlecan is still synthesized as a HSPG [48] because of the HS attachment site in domain V [31]. The *hspg2^{Δ3/Δ3}* mouse shows defective lens capsule formation [47], delayed wound healing and impaired angiogenesis [49], increased smooth muscle cell proliferation [50] and abnormal glomerular filtration [48] but no significant cartilage or bone defects. This suggests either that the single HS chain on domain V is sufficient for growth factor (i.e. FGF-2) action in the growth plate or that, as the normal-length bones of the FGF-2 knockout indicate [20], FGF-2 is not a crucial ligand for FGFR-3 and, thus, for endochondral ossification in the growth plate. In contrast, the FGF-18 knockout has defects in endochondral ossification [24,25] which suggests that FGF-18 is the crucial FGF in long bone growth. FGF-18 binds to the core protein of perlecan. The core protein of perlecan is present in the *hspg2^{Δ3/Δ3}* mouse, which has normal endochondral ossification but is absent in the perlecan null mouse, which has defective endochondral ossification [6,7]. We propose then, from our findings, that the core protein of perlecan may be an important mediator of FGF-18 action in the developing growth plate.

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Acknowledgments

This work was supported by a grant to JRH from Shriners Hospitals for Children

The abbreviations used are

CAF, cationic filtration; CS, chondroitin sulfate; DDSH, Silverman-Handmaker type dyssegmental dysplasia; DMEM, Dulbecco's modified Eagle's medium; DMMB, dimethylmethylene blue; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GAG, glycosaminoglycan; HS, heparan sulfate; SJS, Schwartz-Jampel syndrome.

Fig. 1. Saturation binding of ^{125}I -FGF-18 to growth plate perlecan in the cationic filtration (CAF) assay. ^{125}I -FGF-18 (33 – 2385 ng/ml; 0.75 – 109 nM) was added to tubes without or with perlecan (8 ng GAG/ml). After incubation for 1 h, the samples were filtered across the cationic membrane and the counts retained on the membrane were determined. Specifically bound FGF-18 (specific counts [ie. total counts minus counts bound without perlecan] divided by specific activity and expressed as nanomolar) is shown in the saturation binding curve (Fig. 1A). Scatchard analysis (inset) of the binding data gave a dissociation constant (k_d) of 145 nM. Fig. 1B is an analysis of the lowest five concentrations used in Fig. 1A, including a revised k_d of 27.8 nM based on these five points. Error bars represent $\pm$ S.E.M.

Fig. 2.

Effects of chondroitinase and heparitinase pretreatment of perlecan on ¹²⁵I-FGF-18 binding using the immunoprecipitation (IP) assay. Perlecan (30 ng GAG/ml) was either sham digested (sham), digested with chondroitinase ABC (C), digested with a mixture of heparitinases I and II (H) or digested with both chondroitinase and heparitinases (C+H), incubated with ¹²⁵I-FGF-18 (40 ng/ml) and immunoprecipitated using pre-immune serum or perlecan antiserum and protein G beads. After washing to remove unbound ¹²⁵I-FGF-18, the amount of ¹²⁵I-FGF-18 released from the beads by 2 M NaCl was measured. For each treatment, control values (no perlecan) were subtracted from experimental values (with perlecan) to give our results. Chondroitinase, heparitinase, and chondroitinase + heparitinase treatments of perlecan did not significantly change the amount of ¹²⁵I-FGF-18 released from the beads by 2 M NaCl. Error bars represent ± S.E.M. * denotes significance compared to pre-immune control ($p < 0.05$).

Fig. 3.

Effects of Denaturation, Reduction and Alkylation of Perlecan on $^{125}\text{I-FGF-18}$ Binding in the CAF assay. To determine if core protein structure has an effect on FGF-18:perlecan binding, we used 40 ng/ml of FGF-18 in a CAF assay with 8 ng GAG/ml of perlecan that was either denatured with 8 M urea or both denatured with urea and reduced/alkylated and 50 mM DTT/100 mM Iodoacetamide (all samples were dialyzed after treatment and before the assay). The binding of FGF-18 to perlecan was reduced almost to background binding (– bar), when the core protein structure was additionally altered by reduction and alkylation (Urea/DTT/Iodo bar). * indicates a significant difference from the denatured perlecan ($p < 0.01$).

Fig. 4. Binding of ^{125}I -FGF-18 to native full-length perlecan and to recombinant perlecan domains I-III in the CAF assay. ^{125}I -FGF-18 (Figure 4A: 20 ng/ml, Figure 4B: 160 ng/ml) was incubated with no perlecan (-) or with 10 ng GAG/ml of either full-length perlecan purified from the growth plate or Alt1 (recombinant domains I-III). After 1h, the samples were filtered across the cationic membrane and the counts retained on the filter were determined and divided by the specific activity to determine ng bound. For both perlecan preparations, there were similar percentages (~ 50%) of FGF-18 bound at both the low (Figure 4A) and high (Figure 4B) concentrations of FGF-18 used. Error bars represent $\pm$ S.E.M. * denotes significance compared to control with no perlecan ($p < 0.05$).

Fig. 5. Binding of ^{125}I -FGF-18 to different perlecan domains in the immunoprecipitation (IP) assay. Recombinant perlecan domain III (DIII), domains I-III (Alt1) and domains I-III with no GAGs (Alt2), each at 150 ng/ml, were mixed separately with ^{125}I -FGF-18 (40 ng/ml) and the perlecan was immunoprecipitated with Protein G beads after incubation with pre-immune serum, or with antiserum to perlecan. A control was added where no perlecan was used in the incubation (-). After washing to remove unbound ^{125}I -FGF-18, the amount of ^{125}I -FGF-18 remaining on the beads was determined. ^{125}I -FGF-18 bound to the beads was significantly greater using anti-perlecan serum versus pre-immune serum ($p < 0.05$, paired t-test). There were no significant

differences, however, among the three types of perlecan that were used. Error bars represent $\pm$ S.E.M. * denotes significance compared to control with no perlecan ($p < 0.05$).

Fig. 6.

Effects of FGF-18 and perlecan on growth plate chondrocytes. Chondrocytes were isolated from the lower proliferating (LP), upper proliferating (UP), intermediate (I) and resting (R) zones of fetal bovine rib growth plate cartilage, plated and cultured with or without 25 ng FGF-18/ml DMEM (25F) and with or without 50 ng perlecan GAG/ml DMEM (50P). The DNA content of the cells was measured and the ^3H -thymidine incorporation into DNA determined. Addition of FGF-18 to LP and UP cells caused a significant increase in ^3H -thymidine incorporation (9- and 14-fold, respectively), which was significantly reduced on addition of perlecan. Error bars represent $\pm$ S.E.M. * denotes a significant difference between the bars compared as indicated by the dashed lines ($p < 0.05$).