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ARTICLES

Heparan Sulfate Primed on β-D-Xylosides Restores Binding of Basic Fibroblast Growth Factor

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Abstract Heparan sulfate proteoglycans (HSPG) are obligatory for receptor binding and mitogenic activity of basic fibroblast growth factor (bFGF). Mutant Chinese hamster ovary cells (*pgs*A-745) deficient in xylosyltransferase are unable to initiate glycosaminoglycan synthesis and hence can not bind bFGF to low- and high-affinity cell surface receptors. Exposure of *pgs*A-745 cells to β-D-xylopyranosides containing hydrophobic aglycones resulted in restoration of bFGF binding in a manner similar to that induced by soluble heparin or by heparan sulfate (HS) normally associated with cell surfaces. Restoration of bFGF binding correlated with the ability of the β-D-xylosides to prime the synthesis of heparan sulfate. Thus, both heparan sulfate synthesis and bFGF receptor binding were induced by low concentrations (10–30 μM) of estradiol-β-D-xyloside and naphthyl-β-D-xyloside, but not by *cis/trans*-decahydro-2-naphthyl-β-D-xyloside, which at low concentration primes mainly chondroitin sulfate. The obligatory involvement of xyloside-primed heparan sulfate in restoration of bFGF-receptor binding was also demonstrated by its sensitivity to heparinase treatment and by the lack of restoration activity in CHO cell mutants that lack enzymatic activities required to form the repeating disaccharide unit characteristic of heparan sulfate. Xyloside-primed heparan sulfate binds to the cell surface. Restoration of bFGF receptor binding was induced by both soluble and cell bound xyloside-primed heparan sulfate and was abolished in cells that were exposed to 0.5–1.0 M NaCl prior to the bFGF binding reaction. These results indicate that heparan sulfate chains produced on xyloside primers behave like heparan sulfate chains attached to cellular core proteins in terms of affinity for bFGF and ability to function as low-affinity sites in a dual receptor mechanism characteristic of bFGF and other heparin-binding growth promoting factors.

Key words: heparin, proteoglycans, glycosaminoglycans, receptor-binding, heparinase, chinese hamster ovary cell mutants

Fibroblast growth factors (FGFs) are a family of at least eight structurally related polypeptides characterized by their high affinity for heparin. They are highly mitogenic for mesoderm- and neuroectoderm-derived cells and are among the most potent inducers of neovascularization and mesoderm formation [Burgess and Maciag, 1989; Gospodarowicz, 1991; Folkman and Shing, 1992]. Studies on the mode of action of basic fibroblast growth factor (bFGF) identified a novel role for heparin and heparan sulfate (HS) in the formation of distinct bFGF-heparin/HS complexes that are essential for bFGF receptor binding and activation [Yayon et al., 1991; Rapraeger et al., 1991; Ornitz et al., 1991]. Recently, direct interaction of heparin with a specific sequence in the extracellular domain of the FGF receptor was also identified and suggested to be involved in FGF-induced cell proliferation [Kan et al., 1993]. The crucial role of the cell surface HS was demonstrated by the finding

Abbreviations used: bFGF; basic fibroblast growth factor; BSA, bovine serum albumin; CHAPS, (3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DX, *cis/trans*-decahydro-2-naphthyl-β-D-xyloside; D-xyl, D-xylose; EDX, 3-β-estradiol-β-D-xyloside; GAG, glycosaminoglycans; HEPES, N-(2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; Me_2SO, dimethyl sulfoxide; NX, 2-naphthyl-β-D-xyloside.

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that binding of bFGF to its high-affinity tyrosine kinase receptor is abrogated in mutant Chinese hamster ovary (CHO) cells defective in glycosaminoglycan biosynthesis [Yayon et al., 1991; Ornitz et al., 1992]. Receptor binding and signaling were restored in the presence of exogenously added heparin or HS, regardless of whether intact cells [Yayon et al., 1991; Rapraeger et al., 1991] or soluble recombinant receptors [Ornitz et al., 1992] were used.

Glycosaminoglycan biosynthesis normally occurs on proteoglycan core proteins and initiates through the transfer of D-xylose from UDP-xylose to specific serine residues [Roden, 1980]. Glycosaminoglycan biosynthesis can also occur on exogenous β-D-xylosides, but most xylosides preferentially stimulate chondroitin sulfate synthesis and only weakly prime HS synthesis [Robinson and Lindahl, 1981; Robinson and Gospodarowicz, 1984; Iozzo and Hassel, 1989; Lugemwa and Esko, 1991]. Efficient priming of heparan sulfate (up to 50% of total glycosaminoglycans) was, however, achieved by certain lipophilic β-D-xylosides such as estradiol-β-D-xyloside (EDX) and naphthyl-β-D-xyloside (NX) [Lugemwa and Esko, 1991; Fritz et al., 1994]. In this study we have tested whether these novel xyloside primers can restore the binding of bFGF to glycosaminoglycan-deficient CHO cells transfected with the high-affinity tyrosine kinase receptor for bFGF. We report that HS primed by β-D-xylosides efficiently restores bFGF binding to HS deficient cells, in a manner similar to that induced by soluble heparin or by HS synthesized by wild type cells. This restoration is caused by soluble- as well as by cell-bound HS primed on β-D-xylosides.

MATERIALS AND METHODS

Materials

Recombinant bFGF was kindly provided by Takeda Chemical Industries (Osaka, Japan). Sodium heparin from porcine intestinal mucosa (avg. M_r 14,000, anti-factor Xa 165 IU/mg) was obtained from Hepar Industries (Franklin, OH). Bacterial (*Flavobacterium heparinum*) heparinase I (EC 4.2.2.7) was kindly provided by IBEX Technologies (Montreal, Canada). Dulbecco's modified Eagle's medium (DMEM, 4.5 g glucose/L), Ham's F-12 medium, fetal calf serum (FCS), penicillin, streptomycin, saline containing 0.05% trypsin, 0.01 M sodium phosphate, and 0.02% EDTA (STV) were obtained from Biological Industries (Beit-Haemek, Israel). Tissue culture dishes were from Falcon Labware Division, Becton Dickinson (Oxnard, CA). Twenty-four-well tissue culture plates were from Nunc (Rosklide, Denmark). $Na^{125}I$ and $[^{35}S]H_2SO_4$ were purchased from Amersham (Buckinghamshire, England). Bovine serum albumin (BSA), HEPES, CHAPS, and all other chemicals were of reagent grade and purchased from Sigma (St. Louis, MO). Beta-D-xylosides (NX, 2-naphthyl-β-D-xyloside; EDX, 3-β-estradiol-β-D-xyloside; DX, and *cis/trans*-decahydro-2-naphthyl-β-D-xyloside) were prepared as described [Lugemwa and Esko, 1991; Fritz et al., 1994].

Cells

Wild type Chinese hamster ovary cells (CHO-KI) were obtained from American Type Culture Collection (Rockville, MD). Glycosaminoglycan-deficient CHO mutants, *pgs*A-745 (xylosyltransferase-deficient) and *pgs*D-803 (GlcA-GlcNAc transferase-deficient) were described previously [Esko et al., 1985, 1988; Lidholt et al., 1992]. Mutant 745 cells are deficient in xylosyltransferase, which catalyzes the first sugar transfer step in GAG biosynthesis. The total sulfated GAG produced by these cells is <5% of the amount made by the wild type. Mutant 803 cells produce about 5–10% of the heparan sulfate found in wild type cells, due to a defect in heparan sulfate polymerization. This strain still produces chondroitin sulfate proteoglycans [Esko et al., 1988; Lidholt et al., 1992]. *Pgs*A-745 and *pgs*D-803 cells were transfected with the cDNA for the murine two immunoglobulin-like ectodomain form of bFGF receptor-1 (745-*flg*, 803-*flg*) as described [Yayon et al., 1991; Ornitz et al., 1992]. Cells from stock cultures were seeded into 24-well plates at a density of 2.5×10^5 cells/ml/well in F-12 medium supplemented with 10% FCS, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were maintained at 37°C in a 10% CO_2 humidified incubator. One day after seeding, the medium was replaced by fresh medium containing different concentrations of xylosides. Xylosides were dissolved in Me_2SO and diluted in growth medium prior to their addition to cells. The final concentration of Me_2SO was less than 0.5%.

Labeling and Isolation of Radioactive Glycosaminoglycans

One day prior to radioactive labeling, cells were harvested with trypsin, centrifuged, and washed with sulfate-free Ham's F12 medium

supplemented with 10% dialyzed fetal bovine serum and 100 U/ml penicillin G (labeling medium). Approximately 2×10^6 cells were seeded into 60-mm diameter culture plates and incubated at 37°C in labeling medium. Xylosides were prepared as 100× stocks in DMSO/water (1:1, v/v). [^{35}S]H_2SO_4 (40 Ci/mg) was added to fresh labeling medium (10 μCi/ml) and aliquots were adjusted to different xyloside concentrations. The final concentration of DMSO was 0.5% (v/v) and did not affect GAG synthesis or cell viability. Experiments were initiated by replacing the medium with radioactive medium containing xyloside and the cells were incubated for 3 h at 37°C.

[^{35}S]Glycosaminoglycans were isolated by anion-exchange chromatography using a modification of previously described methods [Bame and Esko, 1989]. Briefly, cells and media were adjusted to 0.1 N NaOH and after 15 min, acetic acid (1 molar equivalent) was added to neutralize the sample. Chondroitin sulfate A (2 mg) and 1/6 volume of a solution containing 1 mg/ml pronase, 1.92 M NaCl, 0.24 M sodium acetate (pH 6.5) were added. After incubating the samples overnight at 40°C, they were diluted 3-fold with water and loaded onto 0.5 ml columns of DEAE-Sephacel (Pharmacia-LKB). GAGs were eluted with 2.5 ml of 1.0 M NaCl in 20 mM sodium acetate buffer (pH 6.0), precipitated by adding 10 ml of cold 95% ethanol and incubating the samples at 4°C for ≥2 h. The samples were centrifuged and the pellets were resuspended in 1 ml of 0.5 M sodium acetate in ethanol/water (1/9, v/v) and reprecipitated with 4 ml of ethanol. The final pellets were dried under vacuum and resuspended in 0.3 ml of 20 mM sodium acetate (pH 6.0). Aliquots (45 μl) of the resuspended pellets were subjected to nitrous acid-catalyzed deaminative cleavage of heparan sulfate as described [Fritz et al., 1994; Bame and Esko, 1989]. The remaining chondroitin sulfate was isolated by anion-exchange chromatography as described above and the amount of [^{35}S]heparan sulfate was determined as the difference between the total [^{35}S]GAG and the remaining [^{35}S]chondroitin sulfate. The amount of recovered material was normalized to the amount of protein in each well.

Iodination of bFGF

Recombinant bFGF was iodinated using chloramine T, as described [Benezra et al., 1992]. Briefly, bFGF (5 μg) was added to 20 μl of 0.2 M sodium phosphate buffer (pH 7.2) containing 1 mCi Na^{125}I. Chloramine T (10 μl of 1 mg/ml) was added for 90 s at room temperature and the reaction was stopped by the addition of 50 μl of sodium bisulfite (1 mg/ml) and 50 μl of NaI (2 mg/ml). The reaction mixture was then applied onto a small (0.1 ml) heparin-sepharose (LKB-Pharmacia) column equilibrated with 0.5 M NaCl, 0.1% CHAPS, 20 mM HEPES, pH 7.4. The column was washed with the same buffer and the ^{125}I-bFGF eluted with 2 M NaCl, 0.1% CHAPS, 20 mM HEPES, pH 7.4. Gelatin was added into the collected ^{125}I-bFGF to a final concentration of 0.02%. The specific activity was $1.2–1.7 \times 10^5$ cpm/ng bFGF and the labeled preparation was stored for up to 3 weeks at −70°C.

Binding of ^{125}I-bFGF

Confluent cultures of CHO cells were washed twice with cold binding medium (DMEM containing 0.1% BSA, 25 mM HEPES, pH 7.4), and incubated (2 h, 4°C) with 5 ng/ml ^{125}I-bFGF in binding medium on a rotary shaker. The cells were washed once with cold binding medium and once with cold PBS containing 0.01% $CaCl_2$ and 0.01% $MgCl_2$. To determine the amount of low-affinity bound bFGF, the cells were incubated (5 min, 4°C) with a solution containing 2 M NaCl and 20 mM HEPES, pH 7.4 [Moscatelli, 1987; Yayon et al., 1991; Benezra et al., 1992]. The incubation medium was counted in a gamma-counter. High-affinity bound bFGF was determined by subsequent incubation (5 min, 4°C) with a solution containing 2 M NaCl in 20 mM sodium acetate, pH 4.5 [Moscatelli, 1987; Yayon et al., 1991; Benezra et al., 1992]. The incubation medium was counted in a gamma-counter. Nonspecific binding, determined in the presence of 200-fold excess unlabeled bFGF, did not exceed 10% of the total binding. At the termination of the binding assay, the cells were fixed in 2.5% glutaraldehyde for 1 h at room temperature, rinsed once with 0.1 M borate buffer (pH 8.2), and stained (45 min, 24°C) with 1% methylene blue in borate buffer. After extensive washing in tapping water, the specific cell incorporated methylene blue was dissolved in 0.1 N HCl (0.5 ml/well, 45 min, room temperature) and determined by its absorbance at 620 nm. Uptake of methylene blue is linearly correlated to the number of viable cells [Goldman and Bar-Shavit, 1979]. The binding data are ex-

pressed as cpm/O.D. (620 nm). All experiments were repeated at least three times.

RESULTS

Restoration of bFGF Binding as a Function of Concentration and Time of Treatment With β-D-Xylosides

CHO cells do not express high-affinity receptors for bFGF but they have abundant low-affinity receptors that represent cell surface heparan sulfate proteoglycans [Yayon et al., 1991]. Mutant *pgs*A-745 cells do not express either high-affinity or low-affinity receptors due to a deficiency in xylosyltransferase which is required to initiate glycosaminoglycan synthesis on proteoglycan core proteins [Esko et al., 1985]. This defect can be bypassed by incubating cells with β-D-xylosides which contain xylose attached in β-linkage to a hydrophobic aglycone [Esko et al., 1987]. The latter facilitates uptake of the xylose, enabling it to act as a primer for the extension of glycosaminoglycan chains. CHO cells produce about 3–5-fold more glycosaminoglycans on β-D-xylosides than on endogenous core proteins and most of the material is secreted [Lugemwa and Esko, 1991; Fritz et al., 1994]. The type of glycosaminoglycan made on a xyloside depends on the structure of the aglycone [Lugemwa and Esko, 1991; Fritz et al., 1994].

To test if β-D-xylosides would restore binding of bFGF to cells, mutant *pgs*A-745 cells were fed with various xylosides. A dose-dependent restoration of low-affinity bFGF binding was obtained in cells that were pretreated with 3-estradiol-β-D-xyloside, reaching a maximum response at 30 μM (Fig. 1). EDX gives rise to a mixture of heparan sulfate and chondroitin sulfate chains [Lugemwa and Esko, 1991]. No restoration of high-affinity binding sites was observed (Fig. 1) since the cells have very few (<1,000) high-affinity receptors.

*Pgs*A-745-*flg* cells transfected with the murine FGF receptor-1 cDNA exhibited little or no binding of bFGF to low- and high-affinity cell surface receptor sites due to the lack of heparan sulfate [Ornitz et al., 1992]. bFGF-receptor binding was restored upon the addition of heparin [Ornitz et al., 1992]. We investigated whether treatment with β-D-xylosides and the associated induction of GAG synthesis can mimic the effect of heparin on bFGF binding to 745-*flg* cells. For this purpose, cells were exposed (48 h, 37°C) to increasing concentrations of three xylosides

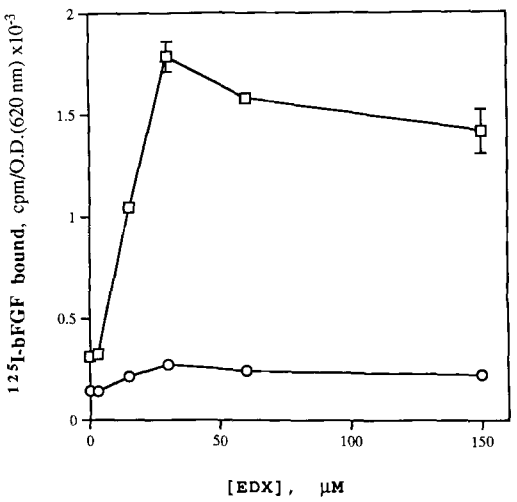

Fig. 1. Effect of EDX on bFGF binding to xylosyltransferase deficient *pgs*A-745 cells. *Pgs*A-745 cells (devoid of high affinity bFGF receptors) were treated (48 h, 37°C) in complete F-12 growth medium with increasing concentrations of EDX. The cells were washed twice with binding medium and incubated (2 h, 4°C) with 5 ng/ml ^{125}I-bFGF in binding medium. bFGF binding to low-affinity (□) and high-affinity (○) binding sites was determined as described in Materials and Methods. Each data point represents the mean ± S.D. of four wells.

(EDX; *cis*/*trans*-decahydro-2-naphthyl-β-D-xyloside [DX]; and naphthyl-β-D-xyloside [NX]) washed free of xylosides and tested for their bFGF binding capacity. A 3–5-fold increase in bFGF binding to low-affinity (Fig. 2A) and high-affinity (Fig. 2B) receptor sites was obtained, depending on the type and concentrations of xyloside applied. High restoration of bFGF binding was observed in 745-*flg* cells treated with relatively low concentrations of NX and EDX (10 μM and 30 μM, respectively). DX exerted a similar high restoration of bFGF binding only at 100 μM. A decline in bFGF high-affinity binding capacity was observed in cells exposed to 200 μM EDX (Fig. 2B) due to toxicity [Lugemwa and Esko, 1991].

The ability of the various β-D-xylosides to restore the binding of bFGF to low- and high-affinity sites was also measured in *pgs*D-803-*flg* cells. This strain lacks enzymatic activities required to form the repeating disaccharide unit characteristic of heparan sulfate [Lidholt et al., 1992]. As shown in Figure 3A and B, none of the xylosides restored low-affinity and high-affinity binding of bFGF to *pgs*D-803-*flg* cells. Stimulation of heparan sulfate synthesis on xyloside primers is associated with inhibition of heparan sulfate proteoglycan synthesis in wild type CHO cells [Lugemwa and Esko, 1991]. Figure 3C

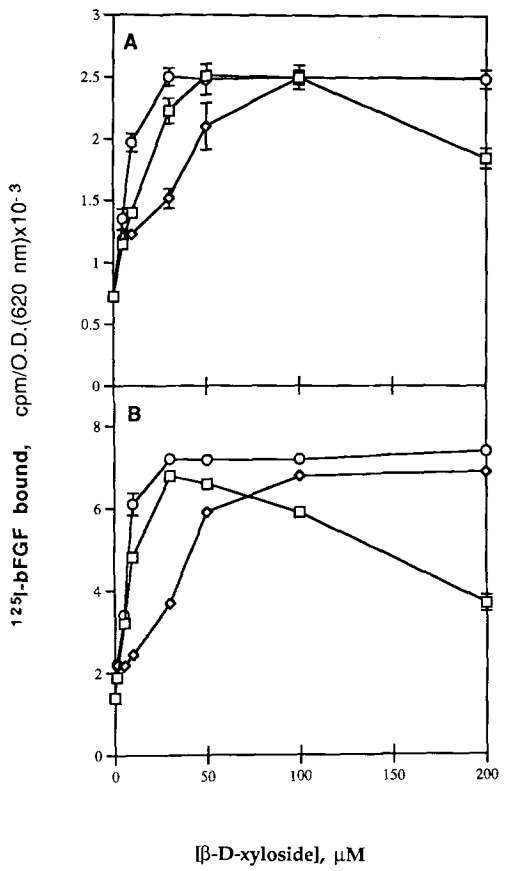

Fig. 2. Dose-dependent effect of β-D-xylosides on bFGF binding to low- and high-affinity receptor sites on *pgs*A-745-*flg* CHO cells. CHO-*pgs*A-745-*flg* cells were treated (48 h, 37°C) in complete F-12 growth medium with increasing concentrations of NX (○), EDX (□), or DX (◇). The cells were washed twice with binding medium and incubated (2 h, 4°C) with 5 ng/ml ^{125}I-bFGF in binding medium. The amount of radioactivity bound to low-affinity (**A**) and high-affinity (**B**) binding sites was determined as described in Materials and Methods. Nonspecific high-affinity binding determined in the presence of 200-fold excess unlabeled bFGF did not exceed 10% of the total binding and was subtracted. Each data point represents the mean ± S.D. of four culture wells. Where error bars cannot be seen, S.D. is smaller than the symbol. The data shown is representative of three experiments.

demonstrates that bFGF binding to low-affinity, presumably heparan sulfate binding sites, was partially inhibited in wild type CHO cells following treatment with NX and marginally affected by treatment with EDX or DX (Fig. 3C). High-affinity binding was not affected by xylosides since untransfected wild type CHO cells do not express high-affinity bFGF receptors (Fig. 3D).

Metabolic labeling, isolation of GAGs, and determination of labeled heparan sulfate were applied to evaluate the effect of xylosides on the synthesis of heparan sulfate by *pgs*-745-*flg* cells. As demonstrated in Figure 4, DX incubated with *pgs*-745-*flg* cells for 3 h in sulfate free medium failed to prime heparan sulfate until a high concentration (50–100 μM) was attained, whereas NX and EDX primed heparan sulfate, up to 50% of the total GAG, at a lower dose (10–30 μM). These results indicate that the ability of NX, EDX, and DX to restore bFGF binding to low- and high-affinity receptor sites correlates with their ability to prime the synthesis of heparan sulfate.

We next studied the kinetics of xyloside-mediated restoration of bFGF binding. For this purpose, *pgs*A-745-*flg* cells were incubated with 30 μM NX (Fig. 5A) or 30 μM EDX (Fig. 5B). At various time periods, the incubation medium was removed and the cells were washed and tested for bFGF binding capacity. Restoration of bFGF binding to both low-affinity and high-affinity binding sites was observed after only 1 h exposure of the cells to xylosides (Fig. 5). A maximal restoration of bFGF binding to low-affinity sites was obtained after 4 h incubation with the xylosides. However, 12 h treatment with NX or EDX was required for maximal restoration of high-affinity bFGF binding (Fig. 5). The results shown in Figures 1–5 indicate that the xyloside-mediated restoration of bFGF binding was i) time and dose dependent, ii) induced by cell- or ECM-associated material since the cells were washed free of soluble material prior to the binding experiment, and iii) depended on heparan sulfate synthesis on the primer.

Restoration of bFGF Receptor Binding Is Induced by Soluble Xyloside-Primed GAG That Bind to the Cell Surface

Glycosaminoglycans produced by β-D-xylosides are mostly secreted from cells. To test if the secreted material would restore bFGF binding, 745-*flg* cells were treated for various time periods with 30 μM NX (Fig. 6A) or 30 μM EDX (Fig. 6B) and the medium was collected and transferred onto untreated 745-*flg* cells. As shown in Figure 6, low- and high-affinity binding of bFGF to untreated cells was restored by medium taken from EDX- and NX-treated cells. A maximal response was induced by medium taken from cells that were treated with xylosides for 10 h. The efficient restoration of bFGF-receptor binding by medium conditioned by β-D-

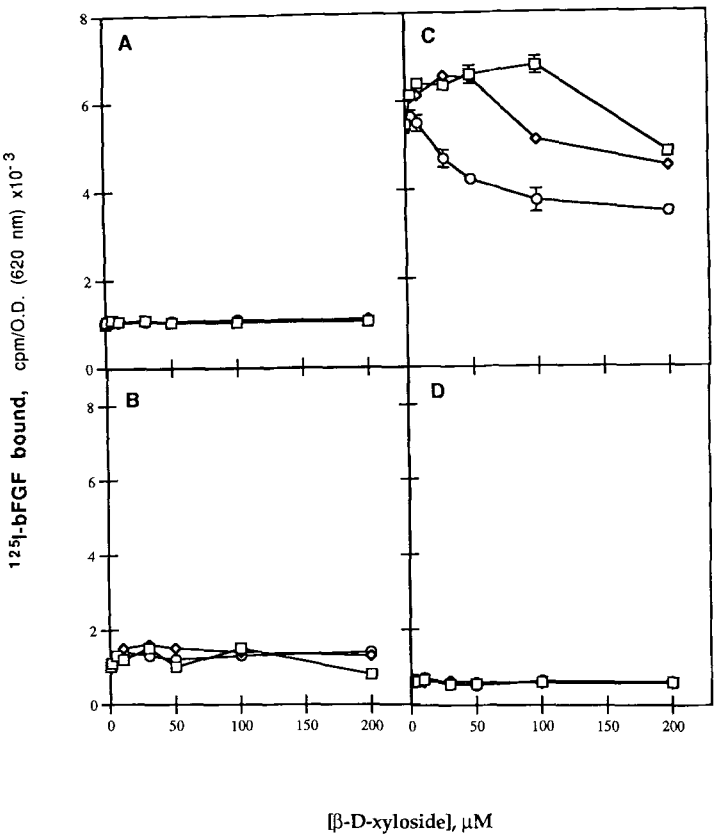

Fig. 3. Dose-dependent effect of β-D-xylosides on bFGF binding to *pgs*D-803-*flg* and wild type CHO cells. **A, B:** *pgs*D-803-*flg* CHO cells. **C, D:** Wild type CHO cells. Cells were treated with increasing concentrations of NX (○), EDX (□), or DX (◇) followed by measurements of bFGF binding to low-affinity (A,C) and high-affinity (B,D) receptor sites, as described in the legend to Figure 1. The variation between different determinations did not exceed ±10% of the mean.

xyloside treated 745-*flg* cells suggests that soluble, newly synthesized xyloside-primed HS chains bind to 745-*flg* cells and act as low-affinity receptor sites for bFGF.

To further demonstrate and characterize this type of interaction, xyloside treated 745-*flg* cells were washed, incubated (20 min, 37°C) with increasing concentrations of NaCl, and tested for their ability to bind bFGF to low- and high-affinity binding sites. While exposure to 0.15 M NaCl had no effect, bFGF binding to EDX- and NX-treated cells that were exposed to 0.6 M and 1.0 M NaCl was reduced (Fig. 7) almost to the level of control cells not exposed to xylosides (untreated). Binding of bFGF to untreated 745-*flg* cells (Fig. 7) and to wild type CHO cells was not affected by preincubation with 0.6–1.0 M NaCl. A similar restoration of bFGF binding was induced upon the addition of heparin (200 ng/ml, 50 ng/2.5 × 10^5 cells/well) to untreated

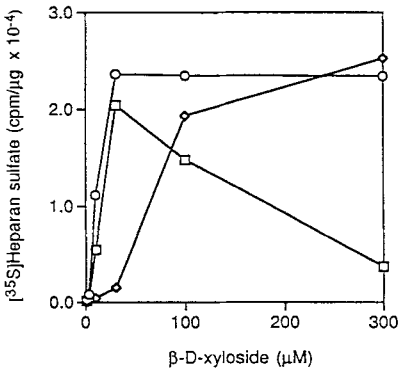

Fig. 4. Dose-dependent effect of β-D-xylosides on heparan sulfate synthesis by *pgs*A-745 CHO cells. Approximately 2 × 10^6 *pgs*A-745 cells were plated into 60-mm diameter dishes. After 24 h, the medium was replaced by sulfate-free F-12 medium containing 10 μCi of $^{35}SO_4$ and different concentrations of NX (○), EDX (□), or DX (◇). The cells were incubated for 3 h, and radioactive heparan sulfate was isolated as described in Materials and Methods.

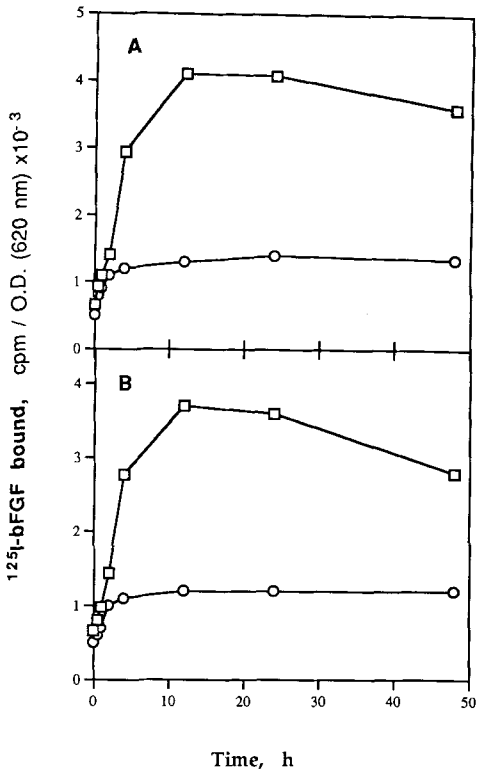

Fig. 5. Time course of β-D-xyloside mediated restoration of bFGF binding. Confluent *pgs*A-745-*flg* cells were treated with 30 μM of NX (**A**), or 30 μM of EDX (**B**) at 37°C for various time periods. The cells were washed twice with binding medium and incubated with 5 ng/ml ^{125}I-bFGF at 4°C for 2 h. The amount of radioactivity bound to low affinity binding sites (○) and high-affinity binding sites (□) was determined as described under Materials and Methods. Nonspecific binding determined in the presence of 200-fold excess unlabeled bFGF did not exceed 50% of the low-affinity binding and 10% of the high-affinity binding, respectively. Each value is the mean of triplicate wells, and the variation between different determinations, did not exceed ±10% of the mean.

745-*flg* cells (Fig. 7). This amount of heparin is similar to the amount of GAG (100 ng/10^6 cells/16 h) that is normally produced by wild type CHO cells or by *pgs*A-745 cells in the presence of β-D-xyloside primers. Restoration of bFGF receptor binding was abolished in 745-*flg* cells that were first incubated with heparin and then exposed to 0.6 M or 1.0 M NaCl (Fig. 7). Complete inhibition of binding occurred when 0.6 M NaCl was present during the binding assay itself. Addition of 0.6 M NaCl following the binding assay had no effect on high-affinity bFGF-receptor binding. Altogether, these results suggest that exogenously added heparin or xyloside-primed GAG bind to the cell surface and restore high-affinity bFGF receptor binding, in a manner similar to that fulfilled by the endogenous cell surface associated HS.

Restoration of bFGF Receptor Binding Is Induced by Heparan Sulfate Primed on β-D-Xylosides

In previous studies we have demonstrated that HS on cell surfaces and in the ECM is highly susceptible to degradation by bacterial heparinase I [Gitay-Goren et al., 1992; Eisenberg et al., 1992]. This enzyme was applied in the present study to analyze the involvement of HS in β-D-xyloside mediated restoration of bFGF binding to 745-*flg* cells. For this purpose, 745-*flg* cells were treated (16 h, 37°C) with 30 μM of DX, EDX, or NX. The incubation medium was removed and the cells were treated with heparinase (0.05 U/ml, 2 h, 37°C) followed by incubation (2 h, 4°C) with ^{125}I-bFGF. No restoration of

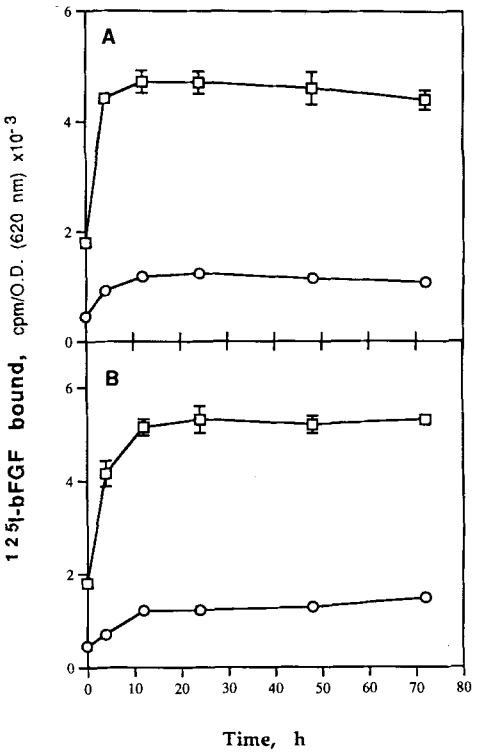

Fig. 6. Restoration of bFGF binding by molecules released from xyloside-treated cells. Confluent CHO-*pgs*A-745-*flg* cells were incubated for various time periods with (**A**) 10 μM NX or (**B**) 30 μM EDX in complete F-12 medium at 37°C. The conditioned media were transferred onto untreated CHO-*pgs*A-745-*flg* cells and the cultures were incubated for 2 h with 5 ng/ml of ^{125}I-bFGF at 4°C. The amount of radioactivity bound to low-affinity binding sites (○) and high-affinity binding sites (□) was determined as described under Materials and Methods. Each data point represents the mean ± S.D. of quadruplicate wells.

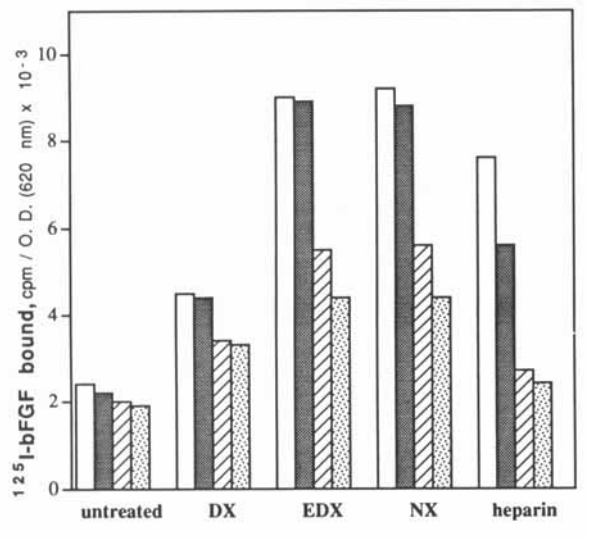

Fig. 7. Effect of salt on high-affinity bFGF binding to xyloside-treated cells. Confluent CHO-*pgs*A-745-*flg* cells were untreated or treated (16 h, 37°C) with 30 μM NX, 30 μM EDX, or 30 μM DX. The media were removed, and the cells were incubated (37°C, 20 min) with 0.15 M (▩), 0.6 M (▨), or 1.0 M (□) NaCl. The medium was not removed from one set of cultures (□) which served as a control. Another set of cells was not exposed to xylosides but was incubated (2 h, 37°C) with 200 ng/ml heparin and subjected to salt treatment as described above. All cells were then washed twice with binding medium and incubated with 5 ng/ml ^{125}I-bFGF at 4°C for 2 h. The amount of radioactivity bound to high-affinity binding sites was determined as described in Materials and Methods. Each data point is the mean of triplicate wells, and the variation did not exceed 10% of the mean.

low-affinity bFGF binding and a significant inhibition of high-affinity bFGF binding occurred when xyloside-treated cells were exposed to heparinase (data not shown). Similar results were obtained when heparin-treated 745-*flg* cells were incubated with heparinase. The inhibitory effect of heparinase was even more pronounced when the medium taken from xyloside-treated 745-*flg* cells was digested with heparinase (0.05 U/ml, 2 h, 37°C) prior to its incubation with untreated 745-*flg* cells. Under these conditions, heparinase treatment completely abolished the restoration of bFGF binding to both low (Fig. 8A) and high (Fig. 8B) affinity binding sites. Heparinase treatment had no effect on cell viability and on the basal low- and high-affinity binding of bFGF to cells that were not exposed to xylosides. These results indicate that species of HS that contain heparin-like sequences are synthesized on the β-D-xyloside primers, resulting in restoration of bFGF receptor binding.

A requirement for both O- and N-sulfation in unique oligosaccharides of heparin and HS was shown to be involved in bFGF binding [Turnbull et al., 1992; Ishihara et al., 1993; Tyrrell et al., 1993]. In the present study there was no restoration of bFGF receptor binding in 745-*flg* cells that were treated with β-D-xylosides in the presence of 30 mM chlorate (Fig. 9), which inhibits GAG sulfation [Bame et al., 1991]. Metabolic labeling of cells with $Na_2{}^{35}SO_4$ revealed that heparan sulfate chains synthesized in the presence of chlorate contained only about 8% of the sulfate present in HS. Unlike the effect of chlorate, inhibition of protein synthesis by cycloheximide (10^{-5} M, 4 h, 37°C) had almost no effect on the ability of β-D-xyloside to restore bFGF binding in *pgs*A-745-*flg* cells (data not shown). This result, together with the inhibitory effect of heparinase and the inability of β-D-xylosides to induce bFGF receptor binding in *pgs*D-803-*flg* cells deficient in HS elongation (Fig. 4A,B), indicates that xyloside-mediated restoration of bFGF

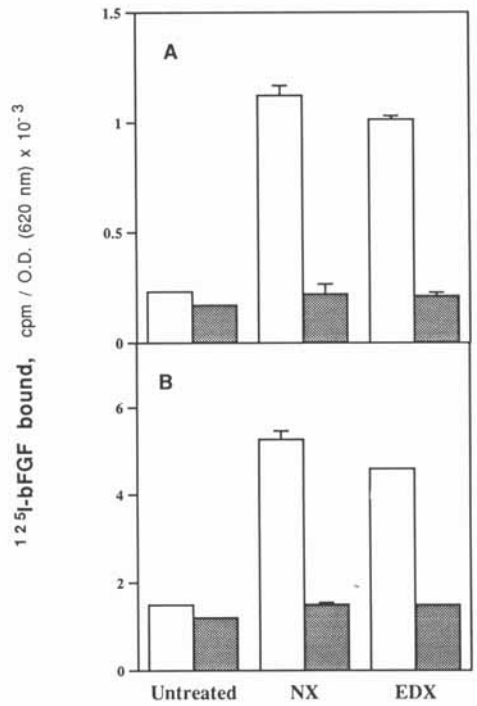

Fig. 8. Effect of heparinase on restoration of bFGF binding by molecules released from xyloside-treated cells. Confluent *pgs*A-745-*flg* cells were treated at 37°C for 14 h with 10 μM NX, or 30 μM EDX in complete F-12 medium. The conditioned media were collected, incubated (2 h, 37°C) with (▩) or without (□) 0.05 U/ml heparinase, and transferred to untreated CHO-*pgs*A-745-*flg* cells. The cultures were then incubated (2 h, 4°C) with 5 ng/ml ^{125}I-bFGF, and the amount of radioactivity bound to low-affinity binding sites (**A**) and high-affinity binding sites (**B**) was determined as described in Materials and Methods. Each value is the mean of triplicate wells, and the variation did not exceed 10% of the mean.

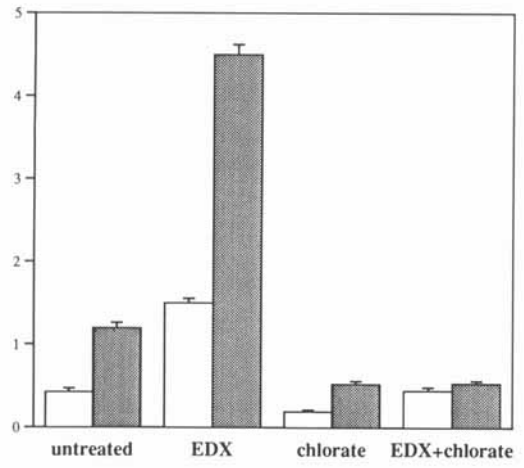

Fig. 9. Effect of chlorate on restoration of bFGF binding to EDX-treated cells. Confluent *pgs*A-745-*flg* cells were treated (48 h, 37°C) with 30 μM EDX, 30 mM chlorate, or 30 μM EDX + 30 mM chlorate. The cells were washed twice with binding medium and incubated with 5 ng/ml ^{125}I-bFGF at 4°C for 2 h. Low-affinity (□) and high-affinity (▨) binding of bFGF was determined as described in Materials and Methods.

binding involves synthesis of HS chains. Moreover, the xyloside primed chains possessed appropriate sequences of sulfated sugars to confer binding and activation of bFGF.

DISCUSSION

Studies of CHO cells defective in GAG synthesis have provided an important tool for exploring the biological activity of proteoglycans in living cells [Esko et al., 1988; Esko, 1991; Shieh et al., 1992]. A recent example is the use of HS-deficient cell mutants to elucidate the obligatory involvement of HS in receptor binding and mitogenic activity of bFGF [Yayon et al., 1991; Ornitz et al., 1992]. These studies showed that HS acts as a cofactor by binding to bFGF and facilitating high affinity interactions with tyrosine kinase receptors on the cell surface [Yayon et al., 1991]. HS mediated stimulation of bFGF receptor binding was attributed to a conformational change in bFGF upon binding of HS, leading to the formation of a stable, receptor-compatible, and biologically active conformation of bFGF [Givol and Yayon, 1992; Prestreslsky et al., 1992]. A more recent study presents evidence that heparin increases the affinity of bFGF for its receptor, but is not required for receptor binding [Roghani et al., 1994]. The distinction between low-affinity and high-affinity bFGF receptor classes, applied in the present study, is based primarily on receptor-ligand dissociation by salt or low pH, respectively [Moscatelli, 1987]. Indeed, Scatchard analysis revealed that this procedure distinguishes between low- and high-affinity binding sites. Comparable results were obtained when low-affinity bound bFGF was released by treatment with heparinase or excess heparin, followed by determination of high-affinity binding by exposure to 2M salt at pH 4.5. Moreover, only low-affinity receptor binding was detected by this procedure in both the sub-endothelial extracellular matrix and untreated 745-*flg* CHO cells. Direct interaction of heparin with a specific sequence in the extracellular domain of the FGF tyrosine kinase receptor was recently demonstrated and shown to be essential for FGF receptor binding and mitogenic activity [Kan et al., 1993]. HS may therefore modulate the conformation of both FGF and its receptor, resulting in the formation of a ternary complex of HS, tyrosine kinase transmembrane glycoprotein, and ligand [Kan et al., 1993].

In the present study we investigated the effect of unique β-D-xylosides capable of priming HS [Fritz et al., 1994] on the binding of bFGF to high-affinity receptor sites expressed by GAG deficient CHO cell mutants transfected with the bFGF receptor. Our results clearly indicate that HS chains synthesized on xyloside primers bind bFGF and enable its binding to high-affinity receptors on the cell surface. In fact, the activity of these xyloside-primed HS chains could not be distinguished from that of soluble heparin or HS normally associated with cell surfaces, indicating that HS chain produced on β-D-xylosides contains the saccharide sequences that cause it to interact with bFGF and its high-affinity receptor. Although most of the xyloside-primed material was secreted from cells, a portion remained associated with the cells. The increase in low-affinity binding of bFGF obtained in the add back experiments indicates that both heparin and xyloside-primed HS bind to the cell surface and this binding is sufficient for restoration of bFGF-receptor binding. Low-affinity binding was independent of *flg* receptors (Fig. 1). It should be noted that the amount of low-affinity sites created in the mutant cells by xyloside priming (Figs. 5, 6) is lower than that normally provided by membrane heparan sulfate proteoglycans in wild type CHO cells (Fig. 3C), indicating that a small amount of cell associated heparan sulfate is needed to restore binding of bFGF to high-affinity receptors. Studies performed with transiently bFGF-receptor transfected CHO cells

[Yayon et al., 1991] revealed a comparable high-affinity binding of bFGF in the presence of the normal amount of cell surface heparan sulfate (receptor transfected wild type CHO-KI cells) or exogeneously added heparin (receptor transfected CHO-803 cells). High-affinity bFGF receptor binding to these cells was similar to that observed with xyloside-treated 745-*flg* cells.

Binding of HS to cells may be mediated by HS binding proteins (e.g., fibronectin, thrombospondin, or type V collagen) [Kaesberg et al., 1989; LeBaron et al., 1988; Castellot et al., 1985]. Binding was disrupted by NaCl at concentrations (0.6–1.0 M) which also disrupt the association between heparin and recombinant FGF receptor [Kan et al., 1993], but are insufficient to dissociate bFGF and heparin. It remains to be elucidated whether heparin and the xyloside-primed HS bind, among other proteins, to the extracellular domain of the FGF receptor tyrosine kinase. It is also of interest to find out whether heparin and the xyloside-primed HS must bind to the cell surface in order to promote bFGF receptor binding, or whether restoration of high-affinity bFGF binding can be induced by soluble species of heparin and HS that bind bFGF under conditions that prevent their interaction with the cell surface.

The dose dependent differences in the ability of various xylosides to prime HS correlated with their ability to induce high-affinity receptor binding of bFGF in GAG-deficient CHO mutant cells. For example, both HS synthesis and bFGF binding were induced by relatively low concentrations of NX and EDX, while DX affected both parameters only at higher concentrations. DX serves as a control for the specificity of priming because it mainly primes chondroitin sulfate at doses where the other primers generate both chondroitin sulfate and heparan sulfate. The maximal proportion of xyloside primed heparan sulfate was about 50% of total glycosaminoglycans. It should be noted, however, that while a high restoration of bFGF receptor binding was observed in 745-*flg* cells treated with 50 μM DX, priming of heparan sulfate synthesis by 50 μM of this xyloside was low. This apparent difference in dose dependency may be due to the different experimental conditions (i.e., 24 h and 3 h exposure to DX in sulfate containing or sulfate free medium) applied in the bFGF binding and heparan sulfate synthesis studies, respectively. Moreover, as discussed above, it is likely that a relatively small amount of heparan sulfate is sufficient to induce a nearly maximal restoration of bFGF receptor binding capacity.

Recent studies indicate that the disaccharide composition of chains generated on NX is similar, although not identical, to that of chains generated on endogenous core proteins [Fritz et al., 1994]. β-D-xyloside primed HS chains may therefore be applied in studies aimed to elucidate the site on HS and type of interaction between heparan sulfate and heparin-binding growth factors. Xyloside-primed HS may also be utilized to modulate cellular responses to bFGF and possibly other heparin-binding proteins. Apart from its involvement in cell interaction with heparin-binding growth factors (i.e., bFGF, VEGF, HB-EGF) [Yayon et al., 1991; Rapraeger et al., 1991; Ornitz et al., 1992; Gitay-Goren et al., 1992; Higashiyama et al., 1991; Ruoslahti and Yamaguchi, 1991], HS may promote cell adhesion and recognition [Kaesberg et al., 1989; LeBaron et al., 1988; Ruoslahti and Yamaguchi, 1991], tumor formation [Esko et al., 1988], as well as binding and uptake of viruses (e.g., herpes simplex virus) [Shieh et al., 1992; Neyts et al., 1992], enzymes (e.g., lipoprotein lipase) [Chajek-Shaul et al., 1990], and lipoproteins [Eisenberg et al., 1992].

Several groups applied defined low Mr and chemically modified species of heparin, as well as bFGF affinity purified species of heparin and HS to determine the structural requirements for interaction between bFGF and heparin/HS. It was found that bFGF binds preferentially to HS regions enriched in Ido A(2-OSO_3) α1-4 GlcNSO_3 and that a minimum size of 8–10 sugar units is required for a heparin-derived oligosaccharide to support the mitogenic activity of bFGF [Aviezer et al., 1994, Ishai-Michaeli et al., 1992; Ishihara et al., 1993; Guimond et al., 1993; Maccarana et al., 1993; Ornitz et al., 1992; Turnbull et al., 1992; Tyrrell et al., 1993]. These data suggested that N-sulfation of glucosamine and 2-O-sulfation of iduronic acid are required for bFGF recognition, whereas 6-O-sulfation does not influence the interaction. The importance of O-sulfation was emphasized in a recent study applying chemically modified species of heparin [Aviezer et al., 1994]. In the present study, a requirement for both heparin-like sequences and high sulfate content was demonstrated by the inhibitory effect exerted by heparinase and chlorate, respectively. As expected, there was no effect to cycloheximide, indicating that protein synthesis and formation of HS core protein are

not involved in xyloside mediated restoration of bFGF receptor binding. Interestingly, xyloside primed chains contain less 6-O-sulfated disaccharide [Fritz et al., 1994], consistent with the finding that 2-O-sulfated residues play a predominant role in bFGF binding [Maccarana et al., 1993; Turnbull et al., 1992]. Further studies are needed to establish the minimal sequence required for binding and biological activation of bFGF.

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