

Stimulation of Fibroblast Growth Factor Receptor-1 Occupancy and Signaling by Cell Surface-associated Syndecans and Glypican

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Abstract. The formation of distinctive basic FGF-heparan sulfate complexes is essential for the binding of bFGF to its cognate receptor. In previous experiments, cell-surface heparan sulfate proteoglycans extracted from human lung fibroblasts could not be shown to promote high affinity binding of bFGF when added to heparan sulfate-deficient cells that express FGF receptor-1 (FGFR1) (Aviezer, D., D. Hecht, M. Safran, M. Eisinger, G. David, and A. Yayon. 1994. *Cell*. 79:1005–1013). In alternative tests to establish whether cell-surface proteoglycans can support the formation of the required complexes, K562 cells were first transfected with the IIIc splice variant of FGFR1 and then transfected with constructs coding for either syndecan-1, syndecan-2, syndecan-4 or glypican, or with an antisense syndecan-4 construct. Cells cotransfected with receptor and proteoglycan showed a two- to threefold increase in neutral salt-resistant specific ¹²⁵I-bFGF binding in comparison to cells transfected with only receptor or cells cotransfected with receptor and anti-

syndecan-4. Exogenous heparin enhanced the specific binding and affinity cross-linking of ¹²⁵I-bFGF to FGFR1 in receptor transfectants that were not cotransfected with proteoglycan, but had no effect on this binding and decreased the yield of bFGFR cross-links in cells that were cotransfected with proteoglycan. Receptor-transfectant cells showed a decrease in glycoporphin A expression when exposed to bFGF. This suppression was dose-dependent and obtained at significantly lower concentrations of bFGF in proteoglycan-cotransfected cells. Finally, complementary cell-free binding assays indicated that the affinity of ¹²⁵I-bFGF for an immobilized FGFR1 ectodomain was increased threefold when the syndecan-4 ectodomain was coimmobilized with receptor. Equimolar amounts of soluble syndecan-4 ectodomain, in contrast, had no effect on this binding. We conclude that, at least in K562 cells, syndecans and glypican can support bFGF-FGFR1 interactions and signaling, and that cell-surface association may augment their effectiveness.

THE signaling pathways that are activated by the binding of various FGFs, Vascular Endothelial Growth Factor (VEGF) and Heparin-Binding EGF-like growth factor to their cognate receptors have been qualified as “heparin dependent.” This contention is based on the failure of these signaling systems in cells that are defective in the synthesis of heparan sulfate (HS)¹ and on the ability to restore the activity of these pathways in these cells by providing an exogenous source of heparin-like polysaccharide. In the case of basic FGF (bFGF or FGF-2), heparin restores the high affinity binding of the growth factor to the tyrosine kinase receptor proteins, and

restores the biological effects of this growth factor on cell differentiation and proliferation (Yayon et al., 1991; Rapraeger et al., 1991). The primary defect in the HS-deficient cells appears to be situated at the level of the initiating event, with the growth factor failing to occupy a binding site on the receptor and to induce a receptor configuration that leads to signaling. Different models that have been proposed as explanations for this HS requirement and the pharmacological effects of heparin (reviewed by Mason, 1994) include: a heparin-induced fit, whereby the glycosaminoglycan allows the growth factor to adopt a conformation that is appropriate for receptor engagement (Yayon et al., 1991), the need for HS to participate in the formation of a multimolecular signaling complex, whereby it binds simultaneously to both ligand and receptor (Nugent and Edelman, 1992; Kan et al., 1993; Guimond et al., 1993; Pantoliano et al., 1994), and indirect effects of heparin on the receptor dimerization that is required for signaling, by promoting the formation of ligand dimers (Ornitz et al., 1992; Spivak-Kroizman et al., 1994). On the other hand, these concepts have also been challenged or

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; GpA, glycoporphin A; GPI, glycosyl phosphatidylinositol; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan.

amended, whereby heparin was shown to only moderately increase the affinity of the growth factor for its receptor (two- to threefold increase) and heparin or HS were proposed only to be needed at low concentrations of ligand (Roghani et al., 1994). In all models, the direct binding interactions between the growth factor and heparin-like glycosaminoglycan are proposed as essential for the activation of the signaling pathway.

bFGF binds preferentially to exon IIIc-containing forms of the FGF receptors (FGFRs) 1–3, which are predominantly mesenchymally expressed (Dionne et al., 1990; Johnson et al., 1991; Keegan et al., 1991; Miki et al., 1992; Yayon et al., 1992; Werner et al., 1992; Chellaiah et al., 1994). In vitro the affinity of bFGF for the IIIc splice variant of FGFR1 is increased by about one order of magnitude when heparin is added (Pantoliano et al., 1994), and in HS-expressing cells, the affinity of bFGF for the receptors (10^{-10} – 10^{-11} M) is about two orders of magnitude higher than the affinity of the growth factor for cell-surface HS (10^{-8} – 10^{-9} M) (Moscatelli, 1987; Wennström et al., 1991). Clusters of IdoA(2-OSO₃) α 1,4GlcNSO₃ units have been identified as bFGF-binding sequences in HS chains derived from human skin fibroblasts (Turnbull et al., 1992) and bovine aortic muscle cells (Habuchi et al., 1992). Heparin-derived penta- or hexasaccharides of similar structure effectively bind to bFGF and inhibit bFGF binding to cell surface HS proteoglycans (HSPGs), but fail to promote FGFR binding (Tyrrell et al., 1993; Maccarana et al., 1993). The minimal structural requirements to enhance bFGF binding to its receptor and to support bFGF-induced mitogenesis appear to be realized in a dodecasaccharide containing the bFGF-binding site and additional 6-O sulfated groups (Ishihara et al., 1993; Guimond et al., 1993; Walker et al., 1994).

The ability of cells to generate HS of a defined sequence complexity varies during ontogenesis (David et al., 1992a; Kato et al., 1994), and some observations directly imply that part of the cellular controls on signaling by FGF-like growth factors may occur at the level of the expression of the required HS cofactor/receptor sequences (Nurcombe et al., 1993). The possibility of PG specificity in this respect is supported by the observation that, in vitro, some heparins and whole PG extracts from human lung fibroblasts were able to induce the binding of bFGF to an immobilized recombinant FGFR–alkaline phosphatase fusion protein, whereas some specific PG forms, such as syndecans 1 and 2, that were purified from these cell extracts were inactive and even inhibited the effect of the active heparin fraction (Aviezer et al., 1994a). In subsequent studies, the major activating component of these PG extracts was identified as perlecan, the large extracellular matrix (basement membrane) PG (Aviezer et al., 1994b). These observations, together with indications that overexpression of syndecan-1 inhibits the bFGF-induced growth promotion of 3T3 cells (Mali et al., 1993), have led to the suggestion that the local expression of active perlecan and the synergies and balances between activating and nonactivating classes of PGs will determine the degree and extent of bFGF-induced cellular responses (Aviezer et al., 1994b).

Our investigations were aimed at identifying cell surface-associated HSPGs that might promote bFGF binding and receptor activation. Using a cell system in which we

were able to express independently the IIIc variant of FGFR1 and four types of PG (i.e., syndecans 1, 2, 4, and glypican), as well as a cell-free system in which we used 6xHis-tailed recombinant forms of receptor and PG to mimic their colocalization at the cell surface, we demonstrate that all these cell-surface HSPGs can support the bFGF–receptor interaction.

Materials and Methods

Plasmid Isolation and Construction

Clones for bFGF and FGFR1 were isolated from a human embryonic lung fibroblast λ ZAPII phage library using oligolabeled PCR-derived probes for bFGF and FGFR1 and standard screening procedures (Sambrook et al., 1989).

The bFGF clones were used as PCR templates. The primer set 5'-GGT-GTCGACATCGAAGGTAGACCCGCTTGCCGAGGATGGC-3' and 5'-GGCTCGAGTCAGCTCTTAGCAGACAT-3' used for the amplification reaction (Saiki et al., 1988) was designed to introduce unique restriction sites flanking the coding sequence and a Factor Xa cleavage site at the amino-terminal end. The PCR products were sequenced using an automated fluorescent sequencer (Pharmacia Biotechnology Benelux, Roosendaal, The Netherlands) and cloned into the prokaryotic expression vector pQE-9 (Qiagen, Chatsworth, CA), which introduces a 6xHis tag at the amino-terminal end of the encoded protein.

One FGFR1 clone, identified as the two Ig-like domain isoform (Ig II/IIIc) (Eisemann et al., 1991), was restricted with PstI to remove 370 bp of the 5' untranslated sequence and cloned into the eukaryotic expression vector pcDNA/Neo (Invitrogen, Leek, The Netherlands), using the HindIII and NotI sites from the multiple cloning sites of the vectors.

The cDNAs for human syndecan-4 (David et al., 1992b), syndecan-2 (Marynen et al., 1989), and syndecan-1 (Mali et al., 1990) were cloned into the KpnI and NheI sites of the episomal expression vector pREP4 (Invitrogen). The cDNA for glypican (David et al., 1990) was released with HindIII and NotI, and cloned into the corresponding sites of pREP4. A 630-bp fragment containing the complete coding sequence of syndecan-4 was antisense cloned into the HindIII and BamHI sites of the same vector.

Plasmids coding for 6xHis-tagged ectodomains of FGFR1 and syndecan-4 were constructed by PCR. A 300-bp fragment of FGFR1 was amplified using the primer set 5'-GACCCGACCCGACATCCAGTGG-3' and 5'-CCGCTCGAGTCAGTGATGGTGATGGTGATGCTCCAGGTACAGGGGCGAGGTCATCATGCC-3', digested with BfrI and XhoI, and cloned into the corresponding restriction sites of the FGFR1 plasmid, replacing the sequences that code for the transmembrane and cytoplasmic domains. The resulting insert, FGFR1e, was cloned into pMEP4 via HindIII and XhoI. A sequence coding for a 6xHis-tagged ectodomain of syndecan-4 was constructed using the primers 5'-GCAAT-TAACCTCACTAAAGGG-3' and 5'-CGCGTCTGACTCAGTGATG-GTGATGGTGATGCTCCGTTCTCTCAAAGATGTTGCTGCCCTGC-3'. The PCR fragment was restricted with BglII and Sall, and cloned in the corresponding sites of the syndecan-4 plasmid. The resulting insert, syn4e, was released with SpeI and XhoI, and cloned into the NheI and XhoI sites of the vector pMEP4. All constructs were sequenced to exclude mismatches.

Purification and Characterization of Recombinant bFGF

Nondenaturing purification of the recombinant 6xHis-bFGF was carried out according to standard protocols (Seno et al., 1990). In short, *Escherichia coli* M15 containing the appropriate pQE-9 construct plus the repressor plasmid pREP4 were induced with 1 mM IPTG at an OD of 0.9. Before sonication for 3 min on ice in the presence of 10 mM β -mercaptoethanol, the harvested cells were incubated for 1 h at 4°C in 50 mM NaH₂PO₄, 10 mM Tris, 300 mM NaCl, 15% sucrose, 0.1 mg/ml lysozyme, and 1 mM PMSF. After centrifugation, the bacterial lysate was applied to an Ni-NTA resin column (Qiagen), equilibrated at pH 8.0 (50 mM NaH₂PO₄, 300 mM NaCl), and eluted at pH 4.5 (50 mM NaH₂PO₄, 500 mM NaCl) (Hochuli et al., 1987). After readjustment to pH 8.0, this eluate was applied to Heparin-Ultrogel (IBF Pharmindustrie, Villeneuve-la-Garenne, France), washed with 1 M NaCl, and eluted with 2 M NaCl. Total yield was ~4 mg purified bFGF per liter of culture (20 g of cells), as determined

by colorimetric assay. The purified protein migrated as a 18-kD peptide in Tricine-SDS-PAGE (Schägger and von Jagow, 1987), and was detectable with an anti-bovine bFGF mAb on Western blot. Stimulation of thymidine incorporation in serum-starved Swiss 3T3 fibroblasts, induced with 2 ng/ml 6xHis-bFGF for 18 h, confirmed the biological activity of this recombinant product.

Extraction and Purification of Cell-surface PGs

Cell surface PGs were extracted with a Triton X-100 buffer in the presence of proteinase inhibitors, concentrated on a DEAE-Trisacryl M column (IBF Pharmindustry, Villeneuve-la-Garenne, France), and further purified by ion exchange chromatography on MonoQ in Triton-urea-Tris buffer (Lories et al., 1987). Immunopurification was carried out with core protein-specific mAbs immobilized on CNBr-activated Sepharose 4B (Lories et al., 1989).

Purification of Recombinant FGFR1 and Syndecan-4 Ectodomains

Recombinant 6xHis-tagged ectodomains of FGFR1 (FGFR1e) and syndecan-4 (Syn4e) were isolated from the conditioned culture media of K562 cells that were transfected with the corresponding episomal plasmid constructs. Serum-free media from pMEP4-transfected cells were harvested 12–16 h after induction with 5 μ M CdCl₂. FGFR1e was purified by two consecutive absorptions on Ni-NTA resin (see above). Syn4e was first absorbed on DEAE-Trisacryl M and then purified by metal chelate chromatography. The final eluates were concentrated by ultrafiltration (Centricon 30; Amicon, Inc., Beverly, MA), and the quantity (~400 μ g/liter medium) and purity (>90%) of the product were estimated by SDS-PAGE.

Western Blotting

Heparitinase and chondroitinase ABC-digested PG were fractionated by SDS-PAGE and blotted on Z probe membranes. The blots were first incubated with the designated mAbs, and then with alkaline phosphatase-conjugated second antibodies, and finally developed with AMPPD (Tropix, Bedford, MA) for chemiluminescence and autoradiography.

Analysis of the GAG Compositions

Free glycosaminoglycan side chains were obtained by proteinase K digestion of purified ³⁵S-labeled PGs. The GAG chains were either subjected to the low pH nitrous acid procedure (Shively and Conrad, 1976) or digested with chondroitinase ABC. Both preparations and an untreated control were precipitated with cetyl pyridinium chloride and then collected on glass filter papers. The HS content was calculated as ($cpm_{untreated} - cpm_{HNO_2}/cpm_{untreated}$), the chondroitin sulfate (CS) content was calculated as ($cpm_{untreated} - cpm_{ABCase}/cpm_{untreated}$). All analyses were performed in duplicates.

Affinity Chromatography of HSPG on Chelate Complex-bound bFGF

Purified 6xHis-bFGF was reapplied to an Ni-NTA column at a concentration of 50–100 μ g/ml gel (~1/100 of the maximal binding capacity), washed with assay buffer (50 mM Na₂HPO₄, pH 7.5, 0.1% Triton X-100, 20 μ g/ml BSA) and increasing NaCl concentrations (0–2 M), and reequilibrated with assay buffer. No bFGF leakage could be detected during the wash. Immunopurified HSPGs were dialyzed against the assay buffer and applied to aliquots of bFGF-Ni-NTA resin. Bound HSPGs were eluted with a NaCl step gradient (0–2 M). Every chromatographic experiment was repeated at least once, with similar results.

Cell Transfections

K562 cells (ATCC CCL 243) were routinely grown in DME F12 medium supplemented with 10% FCS and L-glutamine. For transfection, K562 cells were prewashed with Ca⁺⁺- and Mg⁺⁺-free PBS and incubated for 10 min at 4°C (10⁷ cells/ml Ca/Mg-free PBS) with 30 μ g linearized FGFR1-pcDNA/Neo, or pcDNA/Neo, before electroporation at 240 V and 960 μ F with a gene pulser (Bio Rad Laboratories, Richmond, CA). Selection was started 48 h later with 500 μ g/ml G418. Stable transfection was achieved after 12 d, and subclones were established by two consecutive limited dilu-

tion procedures. Individual clones were characterized for specific ¹²⁵I-bFGF binding. The transfections with the episomal replicons pREP4[–], pREP4[Syn1], pREP4[Syn2], pREP4[Glyp], pREP4[Syn4], and pREP4[antiSyn4] were performed in similar ways. Selection with 200 μ g/ml of hygromycin over 2 wk resulted in stable cell populations that were not further subcloned.

¹²⁵I-bFGF-binding Assays

Iodinated bFGF (specific activity = 800–1,200 Ci/mmol) was purchased from New England Nuclear (Boston, MA), aliquoted directly upon arrival, and stored at –70°C. For the cellular binding assays the K562 transfectants were grown for 72 h in a serum-free medium (DME F12) containing 1 g/liter BSA, 8 mg/l transferrin, and 4 mg/l of insulin, or in Ham's F12 medium supplemented with 30 mM NaClO₃ (to suppress the sulfation of the GAG chains) and the same additives. Samples of 200,000 cells were incubated for 90 min at 4°C in 200 μ l DME F12 supplemented with 1 mg/ml BSA, 25 mM Hepes, pH 7.5, and 10 ng/ml ¹²⁵I-bFGF, in the absence or presence of 1 μ g/ml unlabeled bFGF and with or without 100 ng/ml heparin. The cells were then washed two times with cold PBS and once with 2 M NaCl, 50 mM NaH₂PO₄, pH 7.5. The radioactivities of the salt washes and the cell pellets were counted separately. The values obtained in the presence of 100-fold excess of unlabeled bFGF were considered unspecific binding and were subtracted from the total counts. The data are displayed as the means and SDs of three independent experiments.

In the cell-free binding assay, increasing amounts of ¹²⁵I-bFGF were combined with FGFR1e (6 ng) in the presence or absence of Syn4e (0.8 ng), trypsinized Syn4e (0.8 ng), or heparin (100 ng/ml), in 500 μ l of assay buffer (50 mM NaH₂PO₄, pH 7.5, 150 mM NaCl, 2 mg/ml gelatin, and 0.5% Tween 20). Control mixtures consisted of increasing ¹²⁵I-bFGF or ¹²⁵I-bFGF and heparin concentrations in assay buffer. All mixtures were supplemented with 20 μ l of Ni-NTA resin and incubated on a roller shaker at room temperature for 2 h. Bound label was recovered by centrifugation, washing of the beads in PBS, and discarding of the supernatant. Specific ¹²⁵I-bFGF binding was measured by subtracting the amounts of label bound in control mixtures from the counts associated with the beads in test mixtures. This experiment was carried out three times with two different batches of ¹²⁵I-bFGF. The data were transformed into concentration equivalents and analyzed as Scatchard plots (Scatchard, 1949) using a computer program for linear curve fitting.

Covalent Cross-linking of ¹²⁵I-bFGF to FGFR1

Wild-type K562 cells and FGFR1-transfected K562 cells were prepared and incubated with ¹²⁵I-bFGF, as in the other bFGF-binding studies. After washing with 2 \times 1 ml of cold PBS, the cells were incubated with 100 μ g/ml (0.27 mM) disuccinimidyl suberate (Pierce, Rockford, IL) in PBS at 15°C for 45 min. The reaction was quenched with 20 mM Tris, pH 7.4, in PBS. The cell samples were boiled for 5 min in 2% SDS, 10% glycerol, 20 mM Tris pH 6.8, 1 mM EDTA, and 0.005% bromophenol blue, and were applied on 6–20% polyacrylamide gradient gels. After running, the gels were stained with Coomassie brilliant blue and dried for autoradiography. Quantitative analysis of the intensity of the bFGF-FGFR1 band was performed with an ImageQuant personal densitometer (Molecular Dynamics, Sunnyvale, CA). Reference bands in the Coomassie-stained gels were also measured to exclude differences in loading.

Immunofluorescence Cytometry

Immunocytofluorometry was performed with a FACSort® (Becton Dickinson & Co., Mountain View, CA), and data were analyzed with the program Lysis II. For indirect immunofluorescence staining, K562 cells were incubated with the designated mouse mAbs at a concentration of 10 μ g/ml for 30 min, washed 2 \times , and then incubated with FITC-labeled goat anti-mouse Ab (Nordic Immunology, El toro, CA). Nonreactive, isotype-matched mouse mAbs were used to measure background fluorescence. FITC-labeled anti-glycophorin A (GpA) mAb (clone JC159; Dako Glosstrup, Denmark) and R-phycoerythrin-labeled anti-CD14 mAb (clone TÜK4; Dako) were incubated together and used at the concentration proposed by the manufacturer. Background was determined with correspondingly labeled isotype-matched mAbs (Dual Colour Reagent; Dako). The relative mean fluorescence intensity (rMFI) for GpA was calculated as:

$$\text{GpA rMFI} = \frac{\text{MFI}_{\text{GpA-treated cells}} - \text{MFI}_{\text{background treated cells}}}{\text{MFI}_{\text{GpA-untreated cells}} - \text{MFI}_{\text{background untreated cells}}}$$

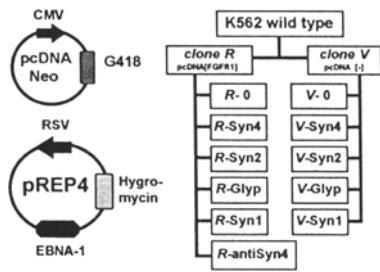


Figure 1. Transfection strategy. K562 cells were first transfected with the integratable vector pcDNA/Neo containing an FGFR1 cDNA or no insert. Two stable subclones, referred to as clone *R* for the FGFR1 transfection and clone *V* for the vector transfection, were then transfected with the episomal vector pREP4, either as such or provided with cDNAs coding for syndecans 4, 2, 1, or glypican (*R-0*, *R-Syn4*, *R-Syn2*, *R-Glyp*, *R-Syn1*, and corresponding *V* cells). In addition, clone *R* was transfected with an antisense syndecan-4 construct (*R-antiSyn4* cells). The cotransfection was realized using the two different selection markers, G418 for pcDNA/Neo and hygromycin for pREP4.

All experiments were performed at least twice; SEM for all MFI values was <4%.

Results

Cell-surface PG Expression in K562 Cells

The transfection strategy that was adopted to study the FGFR system is illustrated in Fig. 1. Wild-type K562 cells, which do not bind bFGF in specific ways (Partanen et al., 1991), lack any transcriptional message for FGFR1 (Armstrong et al., 1992; our own unpublished data), and express only low levels of cell-surface HS (see below), were first transfected with an integratable pcDNA/Neo vector provided with cDNA for FGFR1(IIIc) or without insert. One

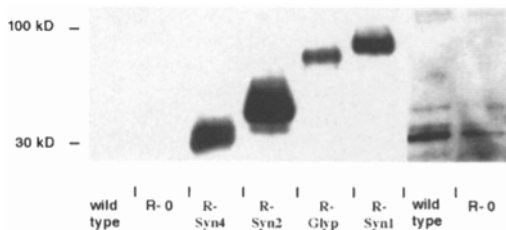


Figure 2. HSPG expression in K562 cells. Triton X-100 extracts of transfected K562, normalized for cell number, were purified by ion exchange chromatography and fractionated by SDS-PAGE after digestion with heparitinase. The Western blot was developed with mAb 3G10, which recognizes the terminal desaturated glucuronate that caps the HS stubs after heparitinase digestion, to detect all proteins substituted with HS, and as an assessment of the number of HS chains that were expressed. Detection was performed by chemiluminescence. Film exposure was given 1 min (first six lanes) or 5 min (last two lanes). PG-transfected K562 populations expressed high numbers of HS chains, which were readily detected after 1 min of exposure, on a single core protein. HS expression was barely detectable in wild-type and *R-0* cells after 1 min, but bands of 120 kD, 50 kD, and mostly 35 kD were visible after the longer exposure. The stronger 35-kD band in these cells reacted with the syndecan-4-specific mAb 8G3 (not shown).

stable subclone from the receptor transfection that showed specific binding of ^{125}I -bFGF (further referred to as clone *R*) and one subclone from the control transfection (further referred to as clone *V*) were then further transfected with the episomal vector pREP4, either as such or provided with cDNA inserts coding for syndecans 4, 2, 1, or glypican to enhance the levels of HS in these cells. Clone *R* was also transfected with a syndecan-4 antisense construct, since this syndecan seems to account (at least in part) for the small amounts of endogenous HS expressed by K562 cells.

After heparitinase digestion, any protein that is substituted with HS can be traced by mAb 3G10, since this antibody recognizes the Δ -glucuronate that caps the HS stubs (David et al., 1992a). In Western blots of PG extracts, this antibody detected several weak bands in wild-type and in *R-0* cells (mainly ~ 35 -kD bands visible after more prolonged exposures), and strong ~ 35 -, 48-, 64-, and 85-kD bands in the *R-Syn4*, *R-Syn2*, *R-Glyp*, and *R-Syn1* transfectants, respectively (Fig. 2). These proteins were positively identified as the expected transfectant proteins with the core protein-specific mAbs 8G3 (syndecan-4), 10H4/6G12 (syndecan-2), S1 (glypican), and 2E9 (syndecan-1) (not shown).

Analysis of the amount of HS expressed at the surface of the transfectants, by quantitative immunofluorescence flow cytometry using the HS-specific mAb 10E4 (David et al., 1992a), revealed marked (5–10-fold) increases in cell-surface HS in all *R*-PG transfected cell populations (Fig. 3, *a* and *b*). The expression of the 10E4 epitope at the surface of *R* antisense transfectants, in contrast, was reduced by $\sim 50\%$ in comparison with *R-0* cells (Fig. 3 *c*). Similar analyses with protein-specific antibodies confirmed the cell-surface expression of the transfectant PGs in transfectant cells, the cell-surface expression of endogenous syndecan-4 in wild-type cells, a >10 -fold increase of the cell-surface expression of the syndecan-4 core protein in *R-Syn4* cells, and the decrease (by 80%) of the cell-surface expression of this syndecan in the syndecan-4 antisense transfectants (not shown). Very similar PG expressions were also achieved in *V*-PG cotransfection experiments (data not shown).

All *R* transfectants were also metabolically labeled with [^{35}S]sulfate for 24 h. PG was extracted from the cells with Triton X-100, and then further purified by ion exchange chromatography on DEAE and MonoQ, as shown for the *R-0* and the *R-Syn4* transfectants in Fig. 4 *a*. Extracts from *R*-PG cells yielded two to fourfold more label per cell than the *R-0* cell extract. The amount of [^{35}S]syndecan-4 recovered by immunoprecipitation from *R-Syn1*, *R-Syn2*, or *R-Glyp* extracts, in contrast, was identical or slightly lower than the amount of [^{35}S]syndecan-4 recovered from *R-0* cells (not shown). Both the *R*-PG and *R-0* materials eluted as a broad early peak (0.45–0.65 M; peak A) and a more distinct later peak (0.70–0.85 M, peak B). All PG transfections lead to increases in both peak A and B materials, but the A/B peak ratio was always higher in *R*-PG extracts than in the *R-0* extract. Qualitatively similar elution profiles were obtained for immunopurified PG (not shown). Endogenous syndecan-4 immunopurified from *R-0* cells mimicked the profile obtained for the total PG extract

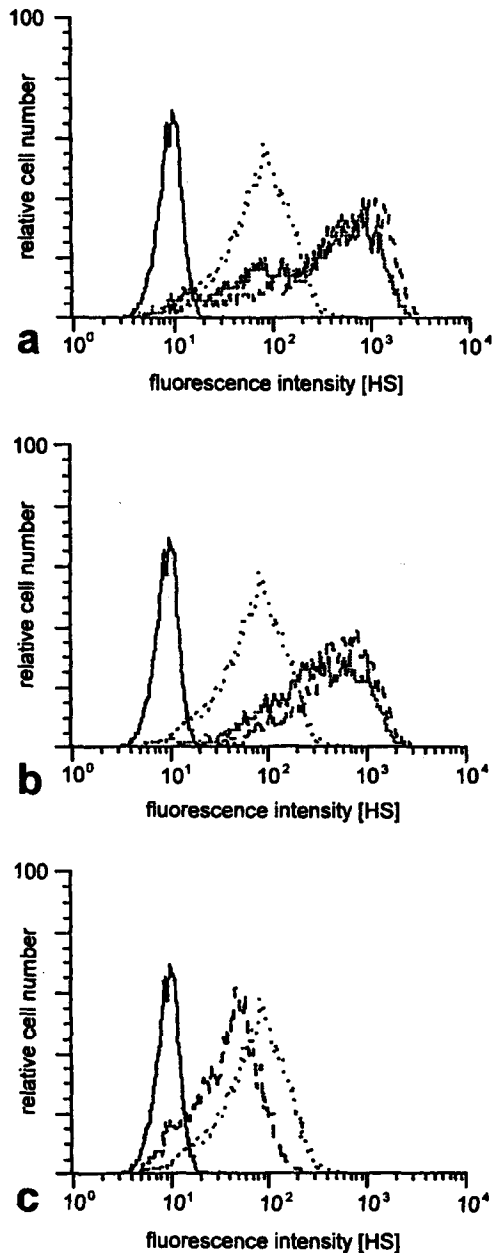


Figure 3. Cell-surface expression of HS in K562 cells. Cell-surface HS was measured by quantitative immunofluorescence cytometry using the HS-specific mAb 10E4. The background fluorescent signal (*solid line*) and the cell-surface HS expression in R-0 cells (*dotted line*), R-Syn1 cells (*narrow dotted line*), and R-Syn2 cells (*dashed line*). (b) The background fluorescence and the cell-surface HS expression in R-0 cells, R-Glyp (*narrow dotted line*), and R-Syn4 (*dashed line*) cells. (c) The background fluorescence and cell-surface HS expression in R-0 cells (*dotted line*) and R-antiSyn4 cells (*dashed line*).

from these cells (prominent B peak), whereas the recombinant PGs and also the endogenous syndecan-4 immunopurified from R-PG cells eluted like total R-PG extracts (more prominent A peak).

Early (A peak) and late (B peak) eluting materials from total extracts were collected as separate pools and used for the further immunopurification of endogenous and/or transfectant PG on the corresponding antibody. Similar

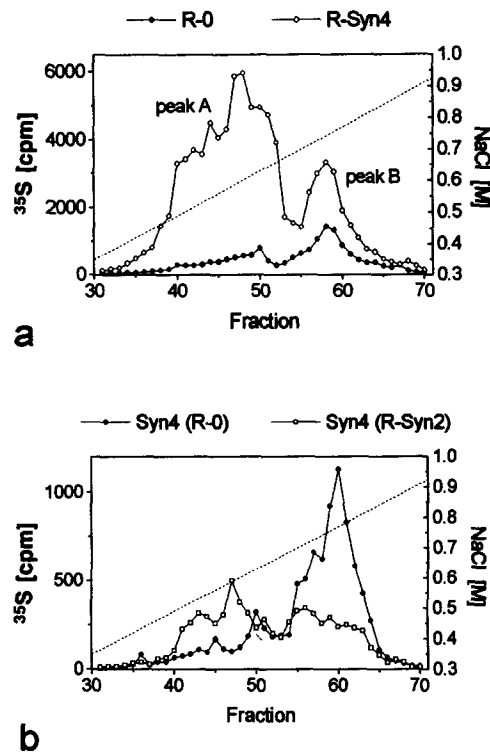


Figure 4. Ion exchange chromatography of the K562 PGs. ^{35}S -labeled PGs produced by the various transfectants were extracted with detergent and subjected to ion exchange chromatography. (a) The MonoQ elution profiles obtained for the total extracts from the R-0 and the R-Syn4 transfectant. The elution profiles obtained for the other PG transfectants were qualitatively similar to the one obtained for R-Syn4. The profile obtained for the R-anti-Syn4 transfectant was similar to that obtained for R-0 cells. (b) The elution profiles obtained for the endogenous syndecan-4 immunopurified from R-0 and R-Syn2 cells after digestion with chondroitinase ABC to remove the CS-substituted forms.

pools were also made for the eluted immunopurified PGs. Analysis of the GAG chain compositions of these immunopurified PGs revealed that the syndecans 1, 2, and 4 isolated from the corresponding transfections contained ^{35}S]HS as well as ^{35}S]CS. This was observed for both A and B peak-derived PGs (with a tendency for a higher HS content in B peak than in A peak materials, 40–60% versus 20–50%). This was also the case for the endogenous syndecan-4 expressed by R-0 cells. Glypican isolated from either the A or the B peak of the R-Glyp extract, on the other hand, carried almost exclusively HS (>90%). The fractionation of intact, heparitinase-, chondroitinase-, and doubly digested immunopurified PG samples by SDS-PAGE analysis and Western blotting indicated that in all instances (except for syndecan-1), the HS and CS chains were present on separate coreprotein populations, with little evidence for hybrid PG (shown for syndecan-4 from R-Syn4 extracts in Fig. 5).

Gel filtration chromatography indicated that the sizes of the protein-free HS chains were nearly invariant (~14 kD), whether isolated from different immunopurified PGs or from peak A or B materials (not shown). Ion exchange chromatography indicated that protein-free HS chains derived from A peaks were less anionic than chains derived

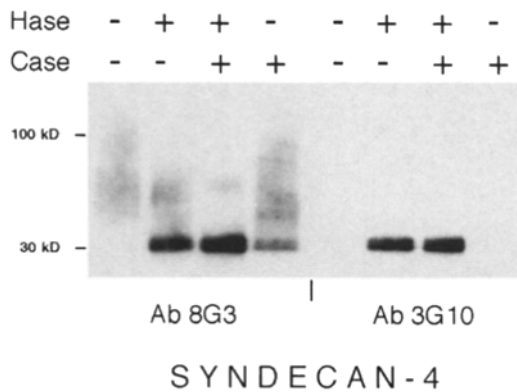


Figure 5. GAG chain composition of the K562 PGs. Immunopurified syndecan-4 derived from the corresponding transfectant (*R*-Syn4) was left untreated (–) or subjected (+) to heparitinase (*Hase*), chondroitinase ABC (*Case*), or both enzymes. The digests were fractionated by SDS-PAGE, blotted, and incubated with the syndecan-4 core protein-specific mAb 8G3 or the anti- Δ -HS mAb 3G10. Comparison of the banding patterns after combined and single enzyme digestions indicated that the majority of syndecan-4 molecules were substituted with HS and a smaller proportion were substituted with CS, with little or no evidence for hybrid molecules. Similar results were obtained for the syndecan-2. Syndecan-1 materials contained higher amounts of CS, in part as true hybrids. Glypican carried almost exclusively HS chains.

from B peaks, but no differences in charge density were observed between HS chains from corresponding peaks derived from different PGs (not shown). Finally, sizing of the different immunopurified cell-surface PGs by SDS-PAGE (after a treatment with chondroitinase ABC to remove the CS-substituted forms) indicated that the more anionic forms of each HSPG species (B peak) were significantly more retarded than the less anionic forms (A peak). Yet, after heparitinase, A and B peak PGs yielded core proteins of similar sizes, indicating increasing numbers of HS-side chains per core protein in the more anionic PGs (not shown).

In another series of experiments, the ^{35}S -labeled *R*-0 and *R*-PG transfected cells were surface biotinylated immediately before the detergent extraction, subjected to ion exchange chromatography, and PG was immunopurified as described above. Early (A peak) and late (B peak) eluting fractions of each PG were then incubated with streptavidin beads to isolate the surface-exposed forms of these PGs. The percentage of streptavidin-bound label did not differ among A and B fractions, indicating that the various forms of a particular PG were equally well represented on the cell surface. This percentage ranged from 60 to 80% for syndecans or glypican isolated from the corresponding PG-transfected cells, but was only ~30% for syndecan-4 isolated from *R*-0 cells (data not shown). From the total label, the HS content, and the size of the biotinylated fraction, it was calculated that the PG transfections resulted in five- to sevenfold increases in cell surface [^{35}S]HSPG.

Altogether, these data demonstrated that overexpression of various cell-surface PGs in K562 cells lead to marked enhancements in cell-surface HS expression. This enhancement was most pronounced for the lesser sulfated forms of this glycosaminoglycan that were present on PGs

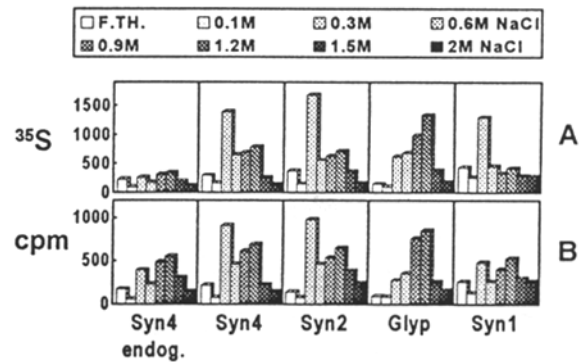


Figure 6. K562 PG binding to immobilized bFGF. Immunopurified, ^{35}S -labeled PGs isolated from peak A and peak B fractions (see Fig. 4) were applied to a bFGF column and eluted with a salt step gradient (up to 2 M NaCl). Glypican eluted as nearly one peak (at 1.2 M), the syndecans eluted as two major peaks, one at 0.3 M and a second at 1.2 M NaCl. The low salt eluates (fall through, 0.1, 0.3, and 0.6 M) contained ~70% CS, and the high salt eluates (0.9, 1.2, 1.5, and 2 M) contained ~90% HS.

of low chain valency, and it occurred at the detriment of the glycanation of the endogenous cell-surface PG (lower average HS sulfation and chain valency). The latter was confirmed by the immunopurification of endogenous syndecan-4 from *R*-0 and *R*-PG cells, digestion of the PG with chondroitinase ABC, and analysis of the HS-substituted syndecan-4 by ion exchange chromatography over MonoQ (Fig. 4 *b*). These findings indicated that the effects of the transfections on HS and HSPG synthesis were not simply additive, but also competitive, somewhat analogous to the effect of β -xylosides on the synthesis of CS (stimulation) and CSPG (inhibition) by cells. They also underscored the conclusion that the gain in cell-surface HS in the transfectants is driven by the transfectant PG.

Cell-surface PGs from K562 Cells Bind bFGF

To evaluate the bFGF-binding properties of the cell surface PGs, the various forms were immunopurified from the corresponding *R*-PG transfectants and allowed to bind to biologically active recombinant bFGF that was immobilized on Ni-NTA agarose via an aminoterminal 6xHis-tag. After equilibration, the column was eluted with an NaCl step gradient (Fig. 6). Syndecans (isolated from A or B peaks) eluted as two major peaks, one at 0.3 M and a second at 1.2 M NaCl. Analysis of the GAG compositions of the eluted syndecan fractions indicated that the pool of the first four fractions (nonbound, and eluting ≤ 0.6 M NaCl) contained mainly CS (~70%), whereas the pool of the four last fractions (eluting ≥ 0.9 M) contained almost exclusively HS chains (~90%). Glypican, which contained only HS, eluted as nearly one peak at 1.2 M NaCl. These data indicate that only HS-carrying forms of the PGs bind significantly to bFGF, and they confirm that most syndecan cores expressed in K562 cells display either HS or CS chains rather than a combination of both.

Heparin Sensitivity of the Binding of bFGF to FGFR1 in K562 Cells

We then measured the binding of ^{125}I -bFGF to PG- and

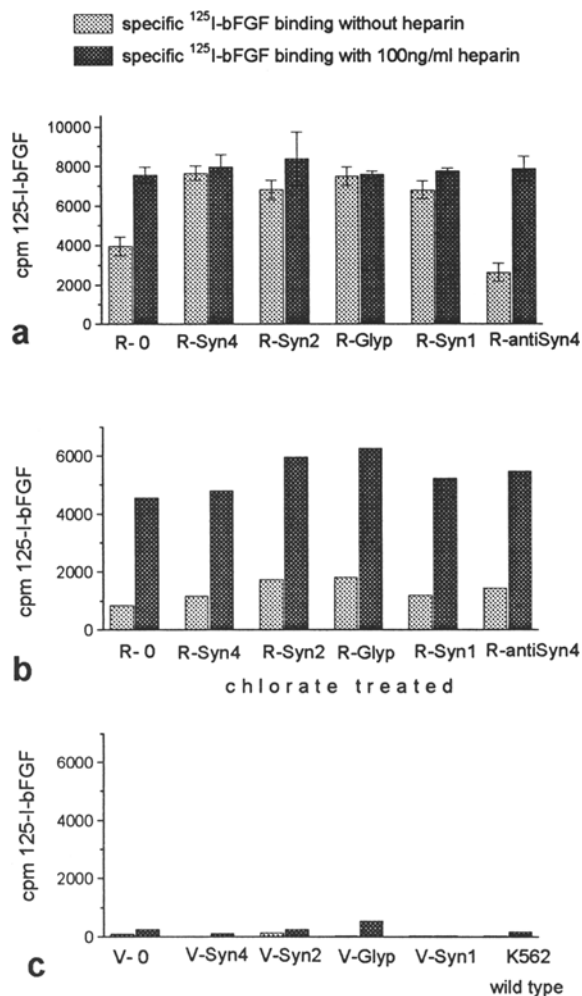


Figure 7. Binding of bFGF to FGFR1 in K562 cells. Aliquots, of 200,000 cells each, were incubated with 10 ng/ml of ^{125}I -bFGF for 90 min at 4°C in the absence or presence of 1 μg /ml of unlabeled bFGF and with or without 100 ng/ml of heparin. The bars indicate the amounts of iodinated bFGF that remained specifically bound after a neutral 2-M NaCl wash of the cells. Results are shown for receptor-transfected cells (a), chlorate-treated, receptor-transfected cells (b), and for non-receptor-transfected cells (c). The values for receptor-transfected cells are given as the means and SDs of three independent experiments.

non-PG-transfected *V* and *R* cells, as well as the effect of exogenous heparin on this binding (see Materials and Methods). Compared to non-PG transfectants, the *V*-PG and the *R*-PG transfectants showed similar (near 10-fold) increases in label in the neutral salt washes of the cells, and this label could barely be displaced by a 100-fold excess of unlabeled bFGF (not shown). All *R*-PG transfectants showed an increase in specific salt-resistant binding of ^{125}I -bFGF when compared with the *R*-0 and the *R*-antiSyn4 transfectants (Fig. 7 a). Adding soluble heparin at a concentration of 100 ng/ml doubled specific bFGF binding to RO cells and tripled specific bFGF binding to *R*-antiSyn4 cells, but had no effect on specific bFGF binding by the *R*-PG transfectant cells (Fig. 7 a). The specific bFGF binding in the presence of heparin was roughly constant for all *R* cell populations (excluding differences in the number of FGFR1

receptors per cell among the various transfectants), and was calculated to correspond to ~30,000 binding sites per cell. Chlorate treatment of all *R* transfectant cell populations resulted in a decrease of the specific binding to 15–25% of the value obtained in the presence of 100 ng/ml of heparin (Fig. 7 b). Neither wild-type K562 cells nor any of the PG transfectants of the *V* clone revealed significant levels of specific ^{125}I -bFGF binding, demonstrating that the assay was measuring FGFR1-related bFGF binding only (Fig. 7 c).

Heparin Sensitivity of the Affinity Cross-linking of bFGF to FGFR1 in K562 Cells

The participation of the cell-surface HS in the bFGF–receptor interaction was also investigated by affinity cross-linking experiments. Covalent cross-linking of ^{125}I -bFGF to the various *R*-transfectants demonstrated a putative bFGF–FGFR1 complex with an apparent molecular mass of ~140 kD. The formation of this labeled complex was inhibited by adding an excess of cold bFGF, and it did not occur in wild-type K562 cells. Quantitative densitometric analysis of the bFGF–FGFR1 complexes in the various transfectants, formed in the presence and in the absence of exogenous heparin, gave the following results: 100 ng/ml of heparin increased the yield of labeled bFGFR cross-links by 26% for *R*-0, by 40% for *R*-antiSyn4, and eightfold for chlorate-treated *R*-0 cells; the same heparin concentration decreased ligand cross-linking by 33% for *R*-Syn4, 46% for *R*-Syn2, 58% for *R*-Glyp, and 23% for *R*-Syn1 (Fig. 8). Increased yields of specific growth factor–receptor complexes in non-PG transfectants and sulfate-starved cells when heparin was added, were consistent with the results from the binding experiments that had revealed an enhancement of the bFGF–FGFR1 interaction by heparin in these cells (Fig. 7). Negative effects of heparin on the yield of growth factor–receptor cross-links in PG transfectants, where heparin did not affect the extent of the specific binding of the growth factor (Fig. 7), suggested modal differences between heparin- and PG-mediated specific bFGF–FGFR1 interactions.

FGFR1 and HS Dependency of the bFGF-induced Block in Erythroid Differentiation of K562 Cells

K562 cells are multipotential malignant hematopoietic cells that spontaneously differentiate into recognizable progenitors of the erythrocytic, granulocytic, and monocytic series. A treatment with hemin or the tyrosine kinase inhibitor herbimycin A reduces the intracellular tyrosine phosphorylation in K562 cells and stimulates their erythroid differentiation (Richardson et al., 1987; Honma et al., 1989). Exposure of K562 cells to 10^{-9} M PMA, in contrast, results in a reduced expression of erythroid-specific proteins, along with a weak myelomonocytic induction (Papayanopoulou et al., 1983). Erythroid differentiation of the K562 transfectants in the presence of growth factor was therefore measured as a test for the functionality of the FGFR1 and to evaluate the possible contributions of cell-surface PG in receptor-mediated growth factor effects.

For these experiments, PG- and receptor-transfected K562 cells were grown in a defined serum-free medium (see Materials and Methods). After 72 h of growth under these conditions, bFGF was added in concentrations of

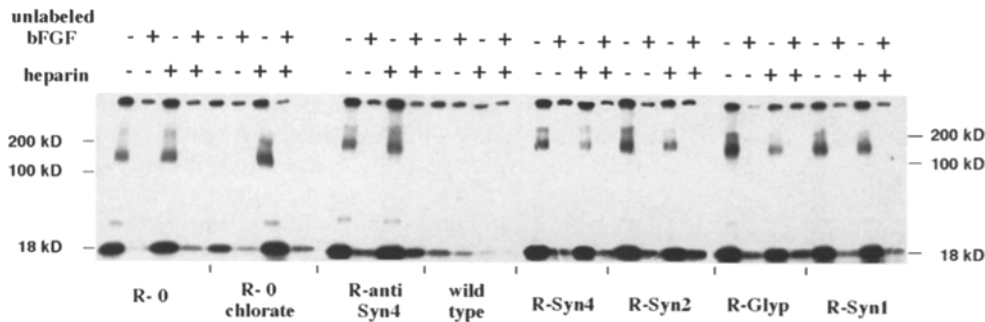


Figure 8. Cross-linking of bFGF to FGFR1 in K562 cells. For affinity cross-linking, the various cell populations were incubated with ^{125}I -bFGF, following the same procedures as described in the legend to Fig. 7. After washing off free label, cell-bound bFGF was cross-linked with 0.27 mM freshly dissolved DSS. The cells were then boiled in SDS

buffer and fractionated by SDS-PAGE. After autoradiography, the intensities of the ~ 140 -kD bFGF-FGFR1 bands were measured with a densitometer. 100-fold excess of unlabeled bFGF eliminated the formation of a labeled bFGF-FGFR1 complex in all receptor-transfected cells. Heparin potentiated receptor cross-linking in non-PG-transfected populations (by 26% in R-0 cells, by 40% for R-antiSyn4 cells) and most strikingly in chlorate-treated R-0 cells (eightfold increase). Wild-type K562 cells lacked any specific receptor cross-linked band. Heparin, on the other hand, decreased the cross-linking of ligand to the receptor in the PG-transfected cell populations (by 33% for R-Syn4, 46% for R-Syn2, 58% for R-Glyp, and 23% for R-Syn1).

0.5–10 ng/ml, and 72 h later, the GpA and CD14 expressions were measured by immunofluorescence flow cytometry. A dose-dependent suppression of GpA was obtained in all these cells (Fig. 9 a). At the high concentration of 10 ng/ml of bFGF, the mean GpA level in all R transfectants was suppressed to approximately one third of the control value (without bFGF), but at lower bFGF concentrations, the PG-transfected R cells were more responsive than the R-0 and the R-antiSyn4 cells. At a bFGF concentration of 10 ng/ml, the CD14 expression was increased by $\sim 50\%$ for all six R transfectants (data not shown). When treated with chlorate, the same cell populations were nearly unresponsive to bFGF, but the effect of bFGF on the GpA expression could largely be restored by the addition of heparin (Fig. 9 b). Neither wild-type K562 cells nor any V transfectant showed a change in GpA or CD14 expression when exposed to bFGF, with or without heparin (Fig. 9 c). Yet, these cells and the R cell populations showed similar decreases in GpA expression in response to 10^{-9} M PMA after 72 h (shown only for wild-type cells in Fig. 9 c). The interpretation that stimulation of FGFR1 increased intracellular tyrosine phosphorylation and consequently blocked erythroid differentiation was supported by the reverting effect of tyrosine kinase inhibitors. In R cells that were preincubated with 30 μM of genistein for 2 h before the addition of 10 ng/ml of bFGF, the GpA and CD14 expressions remained largely unchanged (data not shown).

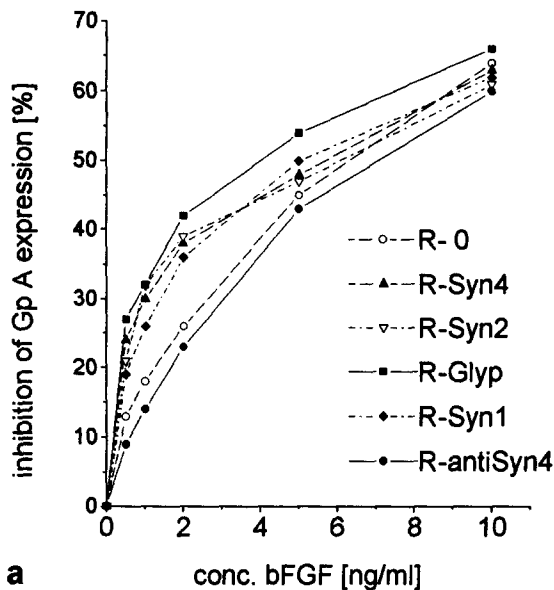
Binding of bFGF to Surface-bound FGFR1 and HSPG Ectodomains

Finally, to exclude possible contributions by non transfectant PGs or other membrane-anchored molecules, we also measured the effect of HSPG on the binding of bFGF to its receptor under cell-free conditions. In this assay, we used recombinant FGFR1e and Syn4e provided with COOH-terminal 6xHis tags that bind with high affinity ($K_d = 10^{-13}$) (Hochuli et al., 1987) to Ni-loaded beads (Fig. 10 a). The affinity of bFGF for the ectodomains was calculated from the label coprecipitated with the Ni-NTA beads versus the free label at various bFGF concentrations (Fig. 10 b). The dissociation constant for the interaction of

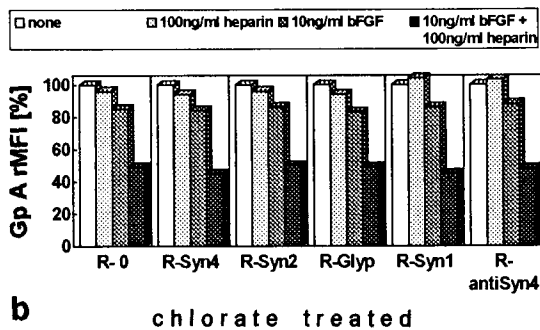
bFGF with Syn4e in the absence of FGFR1e was 2.7 nM (not shown). The calculated dissociation constant for the direct bFGF-FGFR1e interaction in the absence of any source of HS in this assay was 1.8 nM, threefold higher than the dissociation constant for the interaction of bFGF with the combination of FGFR1e and Syn4e (0.6 nM) or the combination of FGFR1e and chondroitinase ABC-treated Syn4e (not shown). In contrast, the affinity of bFGF for the combination of FGFR1e and heparitinase-treated Syn4e was identical to its affinity for FGFR1e. The addition of soluble heparin (100 ng/ml) to bFGF slightly increased the affinity of the growth factor for FGFR1e ($K_d = 1.1$ nM), whereas trypsin-treated Syn4e added at similar concentrations as Syn4e had no effect on the binding ($K_d = 1.7$ nM). For the combination of FGFR1e and Syn4e, the concentrations of the ectodomains were chosen such that the maximal number of bFGF-binding sites contributed by each component were individually similar. Yet the maximal number of binding sites obtained for the combination of FGFR1e and Syn4e did not differ from the maximal number of binding sites obtained for these ectodomains tested individually. This suggested a simultaneous binding of bFGF to both ectodomains, as a ternary complex that has greater stability than that mediated by soluble heparin.

Discussion

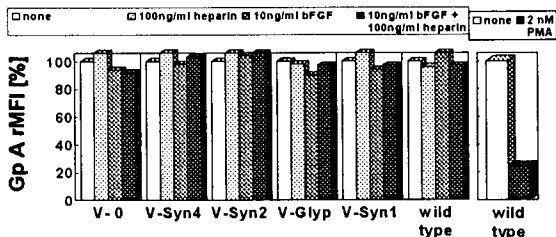
Our results demonstrate that three different syndecans and glypican can promote the binding and activation of a specific kinase receptor form, i.e., the IIIc splice variant of the FGFR1, by a specific member of the FGF family, i.e., bFGF (FGF2), when expressed with the FGFR1 as coreceptor pairs in transfectant K562 cells. All the forms that were tested boost the expression of cell-surface HS in these hematopoietic cells, facilitating the saturation of the receptor with growth factor and increasing the sensitivity of the cells to low doses of the growth factor that inhibit their erythroid differentiation. We conclude that cell-surface PGs can function as partners for the tyrosine kinases in a dual FGFR system, and that several different forms of this category of cell-surface components can provide the source of HS that is required for effective FGF-FGFR bind-



a



b



c

Figure 9. Effect of bFGF on GpA expression in K562 cells. The various K562 cell populations were cultured for 72 h in serum-free medium supplemented with BSA, transferrin, and insulin. After exposure to the indicated concentrations of bFGF for another 72 h in this medium, the cell-surface GpA expression was measured by quantitative immunofluorocytometry. The displayed relative mean fluorescence intensity values were calculated as indicated in Materials and Methods. Receptor-transfected cell populations (*a*) responded with a dose-dependent decrease in GpA expression. At the maximal concentration of 10 ng/ml of bFGF, the GpA was reduced by 60–70% in all transfectants. PG-transfected clones, however, responded at significantly lower bFGF concentrations than *R-0* and *R-anti-Syn4* cells, e.g., at bFGF concentrations as low as 0.5 ng/ml *R-Glyp* still showed a 27% suppression, and *R-anti-Syn4* cells showed only a 9% suppression. Chlorate-treated *R* cells (*b*) were nearly unresponsive

to bFGF, but the effect of bFGF could be restored with exogenous heparin (added at 100 ng/ml). Exposure of the wild-type or the non-receptor-transfected cell populations (*c*) to bFGF in combination with or without heparin did not result in significant changes in GpA mean fluorescence intensity. Treatment of K562 cells with the phorbol ester PMA (2 nM, over 72 h) induced an 80% loss of GpA expression (shown only for wild-type cells in *c*).

K562 Cells as a Model for Studying Cell-surface HS

K562 cells were selected for these studies because a survey of a large panel of cells with the HS-specific mAbs 10E4 and 3G10 had indicated that these cells were able to synthesize authentic HS, a minimal requirement to potentially support bFGF-receptor interactions, but in low and possibly insufficient amounts to support these interactions efficiently. The aim was to test whether transfections with cDNAs coding for cell-surface PGs could compensate for this relative HS deficiency. The results show that after these transfections, K562 cells are capable of expressing ~5–10-fold higher levels of cell-surface HS, and that the endogenous and transfectant cell-surface PGs that account for this HS can be fractionated in distinctive charge and size classes that result from the intrinsic variability of the posttranslational modifications of these proteins. Comparative quantitative immunocytofluorometry indicated that the HS expression in the K562 transfectants reached similar levels as in human lung fibroblasts (not shown), suggesting that these transfectants provide relevant models for the display of cell-surface HS in constitutive high expressors. It may be significant, however, that the gain of HS in these cells is more pronounced for the PG fractions that elute early from MonoQ (substituted with fewer and less sulfated chains) than for those that elute later in the salt gradient (substituted with more and more highly sulfated chains) (Fig. 4). Together with the reduced levels of HS glycanation of the endogenous syndecan-4 in the transfectants, these results suggest that in K562 cells, individual core proteins compete with each other for a limiting HS glycanation machinery, and that in high expressors, a smaller proportion of the PGs therefore reaches the most extensive levels of substitution and modification. These findings are reminiscent of results obtained for the synthesis of antithrombin III-binding HS sequences in transfectant endothelial and fibroblastic cells, where several consecutive transductions of a syndecan-4 expression vector progressively enhanced the production of core protein and total HS in these cells, but reduced the levels of antithrombin III-binding HS present on transfectant and endogenous PG (Shworak et al., 1994). This suggests that the production of defined HS sequences can be saturated and that the specific activities of the PGs in terms of these sequences depend at least in part on the core protein expression levels. In the K562 PG transfectants, the transfectant cores drive the synthesis of ~90% of the cell-surface HS, but these expression levels still appear compatible with the production of fully modified forms of PG and the produc-

tion of fully modified forms of PG and the produc-

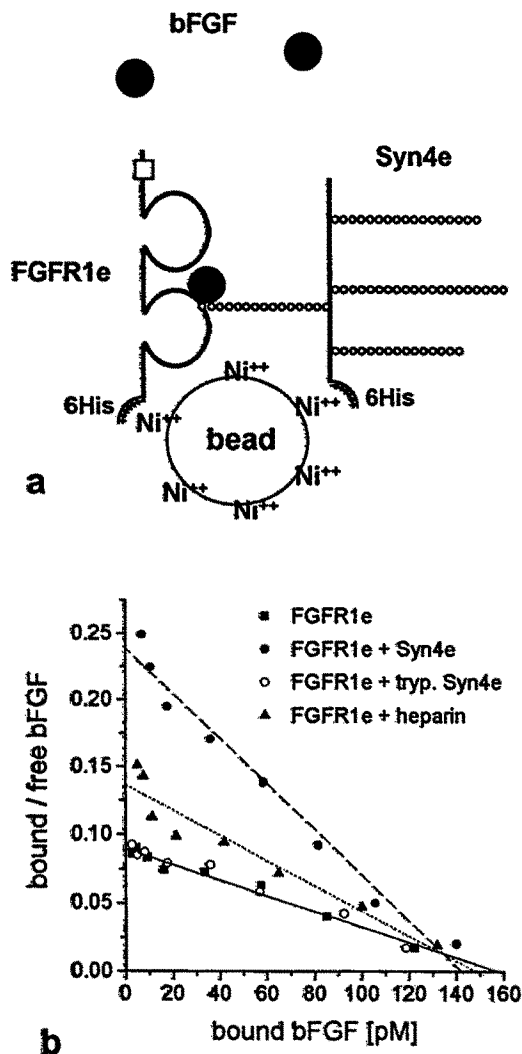


Figure 10. Coimmobilized HSPG increases the affinity of bFGF for the ectodomain of FGFR1. Plasmids coding for COOH-terminal 6xHis-tailed forms of the ectodomain of FGFR1 (*FGFR1e*) and syndecan-4 (*Syn4e*) were expressed in K562 cells. These 6xHis tags bind with high affinity ($K_d = 10^{-13}$) to Ni-loaded beads (a). Binding of radiolabeled bFGF to Ni-NTA-immobilized FGFR1e in the presence or absence of *Syn4e*, trypsinized *Syn4e* (which lacks the 6xHis tag and therefore can not bind to the Ni-NTA resin), or heparin was measured at varying concentrations of growth factor (b). The affinity of the binding was calculated from the specifically bound versus free radiolabel at various bFGF concentrations. The data are depicted as Scatchard plots. The concentrations of FGFR1e and *Syn4e* used were such that the maximal number of bFGF-binding sites provided by each component individually (as determined from separate binding experiments) were identical.

tion of the sequences required for bFGF binding and activation. It is conceivable, however, that similar transfections in cells that express large near-saturating amounts of endogenous HSPG could have adverse effects on the synthesis or colinearity of the sequences that are required for bFGF activation, and that this competition might explain how the expression of syndecan-1 in 3T3 cells suppresses the growth response of these cells to bFGF (Mali et al., 1993).

Facilitation of bFGF–Receptor Binding by Cell-surface HS

The effects of chlorate on the binding of bFGF to *R* transfectants and of heparin on chlorate-treated *R* transfectants were consistent with the observations of several other investigators, suggesting a clear HS-dependency of the specific binding of bFGF to FGFR1(IIIc) and indicating that K562 cells were able to produce the HS sequences that are required for the stimulation of this binding. Heparin, however, also enhanced the levels of specific bFGF binding in *R-0* cells that were not treated with chlorate, indicating lack of receptor saturation in these cells despite normally saturating concentrations of added growth factor, possibly caused by PG receptor imbalances in these receptor-over-expressing cells. The saturation of the receptor in the *R-PG* transfectants confirms this interpretation and indicates that all the different cell-surface PGs tested can complement for the relative HS deficiency of these cells. Reduced levels of receptor saturation in *R-anti-syndecan-4* transfectants in comparison to *R-0* cells support the contention that cell-surface PGs contribute to receptor binding in K562 cells, in apparent discrepancy with previous suggestions that these forms are inactive or even inhibitory in this respect (Aviezer et al., 1994a). Distinctive PG requirements for activation of the receptor in *cis*- and *trans*-modes, or unique activities of the PGs in these cells, could account for this discrepancy.

Our cell-free assay demonstrates that bFGF binds to the ectodomain of the two-Ig domain form of human FGFR1 in the absence of heparin, and that heparin moderately enhances the affinity of this binding interaction, which agrees with the results reported by several other investigators with similar constructs (Kiefer et al., 1991; Bergonzoni et al., 1992; Roghani et al., 1994). In this assay, a syndecan ectodomain made by K562 cells that could be coimmobilized with receptor proved to be an effective strengthener of the binding interaction, whereas the same ectodomain provided in equimolar amounts, but in soluble form, had no detectable activity. The failure of these soluble ectodomains is in agreement with previous binding results obtained for receptor-reporter fusion proteins and soluble cell-surface PG in cell-free assays (Aviezer et al., 1994a) and for the activation of receptor in HS-deficient cells by exogenously added PG (Aviezer et al., 1994b), whereby several of the cell-surface PGs that were studied here were proven to be ineffective. All together, these findings suggest that cell-surface PGs are not intrinsically ineffective, but that membrane-imbedded and solubilized forms of the PGs from a particular cell differ in their activities on bFGF–receptor binding in that the former lead to higher effective concentrations of reactants with higher apparent binding affinities as a result.

The results from the affinity cross-linking experiments are also consistent with a role for cell-surface HS in the receptor–ligand interaction. They show specific receptor binding in *R* cells and an increase in receptor–cross-linked ^{125}I -bFGF for the *R-0* and chlorate-treated *R-PG* cell populations upon the addition of heparin, consistent with the stimulatory effect of heparin on receptor occupancy in these cells. Somewhat surprisingly, since heparin did not promote or decrease receptor occupancy in *R-PG* trans-

fectants, heparin very consistently decreased the cross-linking efficiency in all R-PG cells. This difference in receptor–ligand cross-linking efficiency between PG-mediated and (in the presence of a large excess of heparin) probably heparin-mediated receptor–ligand complexes suggests the formation of distinctive receptor–ligand complexes in the two situations. Cross-linking likely involves sites within the bFGF–receptor complex other than those directly involved in the binding interaction, and depends on the configuration of the complex, the realized approximations, and the stability of the complex. Conformational changes induced by exogenous heparin, but not by HS, that may be irrelevant for binding might disturb bFGF cross-linking. The finding that ternary complexes mediated by surface-immobilized ectodomains are more stable than heparin-mediated complexes might also be relevant and relate to the reduction in FGFR–FGF cross-links in the presence of heparin. The observation at least suggests that exogenously added heparin does not exactly reproduce the process of PG-mediated binding and cannot be used as the sole model to define the molecular requirements for receptor occupancy by growth factor and activation.

Receptor Activation by Cell-surface HS

The fact that K562 cells that expressed high levels of cell-surface HS, (R-PG cells) responded more clearly to low bFGF concentrations than low expressors, (R-0 and R-anti-Syn4 cells) further supports the contention that, at low doses of growth factor, ligand-induced FGFR stimulation and signaling depend on the availability of sufficient and appropriate sources of HS at the cell surface (Roghani et al., 1994). Our data show that several different cell-surface PGs originating from separate molecular families are able to provide this source, at least for the bFGF–FGFR1 interaction, in cells with the appropriate HS-synthesizing machinery, and when expressed at the cell surface of the receptor-expressing cells. Relatively low specific activities of the cell surface PGs with respect to the fostering of receptor–growth factor interactions may be compensated by this membrane association, essentially limiting the activity of these PGs to the *cis* mode. Considering the relative ineffectiveness of the soluble PG ectodomains as promoters of the bFGF–receptor interaction, it may be significant that all syndecans have conserved a putative protease cleavage site in their ectodomain, and that glypicans are linked to the cell surface by phospholipase-susceptible bonds. Syndecan shedding is known to occur, at least under *in vitro* conditions, and phospholipase D activities that release soluble bFGF–HSPG complexes have recently been identified in HeLa cell and bone marrow stromal cell cultures (Metz et al., 1994). From our results, we would predict that at low concentrations of growth factor, protease- and lipase-induced sheddings of the cell-surface PGs will lead to a dilution of the reactants, dissociation of the receptor complexes, and downregulation of the signaling pathway, unless other PGs with possibly unique *trans*-activation potentials, such as the perlecan synthesized by cultured fetal lung fibroblasts (Aviezer et al., 1994b), can compensate for this loss. This leads to the speculation that PG shedding may provide means for acute regulation of *cis*-activated heparin-dependent pathways, next to possibly slower regula-

tions via controls on the synthesis of the core proteins and the required HS sequences.

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