Membrane Heparan Sulfate Proteoglycan-supported FGF2-FGFR1 Signaling

EVIDENCE IN SUPPORT OF THE “COOPERATIVE END STRUCTURES” MODEL*

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Received for publication, July 13, 2001, and in revised form, September 7, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M106688200

Fibroblast growth factor 2 (FGF2)-initiated FGF receptor (FGFR)-signaling requires the assistance of heparin/heparan sulfate. Here, we evaluated the effects of different heparan sulfate proteoglycan (HSPG)-expressing cell lines and HSPGs derived from these cells on FGF2-induced FGFR1-phosphorylation in heparan sulfate-negative BaF3 cells. HSPGs supplied in membrane-associated form, by presenting cells, were all effective promoters of FGF2-initiated FGFR1 phosphorylation, independently of their nature (syndecan/glypican) or cellular origin (human lung fibroblasts, transfected Nalmalwa cells, or transfected K562 cells). A treatment with heparitinase initially stimulated, but finally completely inhibited, the activity of these presenting cells. In comparison, equivalent amounts of soluble HSPGs, obtained by trypsinization of these cells or by immunopurification from cell extracts, did not promote FGF2-induced FGFR1-phosphorylation, yet removal of the less anionic species or a further treatment with heparitinase converted these soluble fractions into potent activators of FGF2-FGFR1 signaling. Extrapolating from current structural models, we suggest that FGF2 dimerization and autophosphorylation is supported by cooperative “heparin-like end structures,” and that cell surface association and concentration compensate for the relative scarcity of such end structures in native HSPGs. In this model, “proteolytic” shedding of heparan sulfate would act as a diluting, down-regulatory mechanism, while “heparanolytic” shedding might act as an up-regulatory mechanism, by increasing the concentration of these end structures.

Fibroblast growth factors (FGFs)1 bind not only to their cognate receptors (FGFRs) but also to heparan sulfate proteoglycans (HSPGs). HSPGs are associated with the cell surface of many, if not most, cell types. Most known HSPG functions are contributed by the interactions of the heparan sulfate (HS) chains of these molecules. Besides growth factors, these HSPGs interact with various adhesion molecules, protease inhibitors, and enzymes, modifying the spatial distributions and activities of these ligands. Among FGFs, the interaction with FGF2 has been studied most intensively, and it is now generally accepted that HSPGs play important roles in FGF2 signaling. Initially, the association of FGF2 with HS has been proposed to protect this FGF from proteolysis and thermal denaturation (1, 2) and to serve as a reservoir of growth factor that can be released by enzymes that degrade the proteoglycans (1). Later, HSPGs were identified as co-receptors for FGF2, strongly promoting FGF-FGFR binding and the subsequent activation of the receptor (3, 4). Recently, genetic studies in Drosophila provided compelling evidence that HSPGs are essential for FGF signaling in vivo (5).

Although the importance of HSPG in FGF-signaling is well documented, the nature of the “co-receptor” and the precise mechanisms at work are less well characterized. Distinctive core protein structures define two major families of cell-surface-associated HSPGs: syndecans and glypicans (6). Prior work from our laboratory showed that syndecans and glypican-1 stimulate FGF2-FGFR1 interaction and signaling in K562 cells, at least when co-expressed with receptor in these cells (4). This agrees with the work of other groups, showing that cell surface syndecan-1 from Raji cells acts as a positive regulator of FGF2 binding and signaling (7), that syndecan-2 on human macrophages promotes FGF2-mediated proliferation (8), and that glypican-1 can stimulate FGF2 signaling (9, 10). Meanwhile, there are also other, contradictory reports. Syndecans and glypicans purified from human lung fibroblast extracts are unable to promote high affinity binding of FGF2 to FGFR1 (11), overexpression of syndecan-1 in NIH 3T3 cells inhibits FGF2-induced proliferation (12), and HSPGs purified from endothelial secretions prevent FGF2 binding to vascular smooth muscle cells and inhibit FGF2-induced mitogenesis (13). One possible explanation for this discrepancy is that the HSPGs were from different sources and might have had different compositions. It is well known, indeed, that the fine structure of HS is cell- and differentiation-specific, and highly diverse (14, 15).

Another possibility is that the membrane association of HSPG might play a role in promoting FGF2 signaling, since most of the inhibitory effects of HSPGs reported so far relate to soluble forms. Syndecans and glypicans are constitutively shed from cultured cells (16, 17), and shed soluble syndecan ectodomains can also be found in inflammatory fluid (18), where they appear to act as inhibitors of FGF2 (19). Finally, sometimes different end point analyses were used as a measure of receptor activity. In the present study, we attempted to define the relative importance of these variables. We used FGF2-induced FGFR1
autophosphorylation as an end point and tested the activities of HSPGs from different origins, both in soluble and in membrane-associated form.

EXPERIMENTAL PROCEDURES

Plasmid Isolation and Construction—Clones encoding FGFR1 were isolated from a human embryonic lung fibroblast λ ZAP II phage library (4). The cDNA of the IgVIII isoform of FGFR1 was used as a polymerase chain reaction template. The primer 5'-CGGGAGCGAACCCTCGGTCGGCGGACACGGCGTGGCTG-GAC-3' was designed to introduce a BamHI restriction site at the 5'-end. The BamHI-SpeI fragment of this FGFR1 cDNA was blunt-ended at the SpeI site and cloned into the eukaryotic expression vector pCDH (Invitrogen, San Diego, CA), using the compatible BgII and Smal sites, respectively. The same cDNAs were also cloned in pcDNA3/neo, using the KpnI and NotI sites and HindIII and XhoI sites, respectively.

Chain valence mutants of glypican-1 were generated with the Transformer site-directed mutagenesis kit (CLONTECH Laboratories, Palo Alto, CA). The primer (and corresponding antisense primer) 5'-GACGACGGGCGCGGC-AGGGCAGCGGCGCGGGCGCGGTCGGTATGGCTG was used to generate the two-chain form (changing Ser<sup>400</sup> into Ala<sup>400</sup>) and the corresponding antisense primer 5'-GAGCGAGCCGAGGCGGGCGGC-CCGGTATGGCTG was used to generate the single chain form (changing Ser<sup>400</sup> into Ala<sup>400</sup> and Ser<sup>490</sup> into Ala<sup>490</sup>). The HindIII–XhoI fragments of the mutant cDNAs, blunt-ended at the XhoI site, were cloned into the eukaryotic expression vector pRFp4 (Invitrogen), using the HindIII and blunt XhoI sites.

Cell Transfections—BaF3 cells (generously provided by Dr. D. M. Ornitz) were routinely cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah) and 10% WEHI cell conditioned medium (as a source of murine interleukin-3). Before transfection, cells were washed with calcium/magnesium-free phosphate-buffered saline (PBS). For transfection, 1 × 10<sup>5</sup> cells were incubated with 30 μg of linearized HA-FGFR1-pDisplay or pDisplay plasmid in 0.5 ml of calcium/magnesium-free PBS at 4 °C for 10 min. Cells were then electrotransfected at 350 V and 960 microfarads (Gene Pulser; Bio-Rad). After 24–48 h of cell culture, selection was started with 600 μg/ml G418 (Life Technologies). Two weeks later, stable clones were obtained, and subclones were established after 1 month. Individual clones were tested for HA-FGFR1 expression as described under “Western Blotting.” One of the highest expressors, clone B6, was selected for further experiments.

Namalwa cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. For transfection, 1 × 10<sup>5</sup> cells were incubated with 30 μg of linearized syndecan-1-pCDNA/neo, glypican-1-pCDNA/neo, or pcDNA/neo plasmid in 0.5 ml of calcium/magnesium-free PBS at 4 °C for 10 min. Cells were then electrotransfected at 240 V and 960 microfarads. Selection was started 24–48 h later, with 1.5 mg/ml G418. Stable transfection was achieved after 2 weeks. The selected population was characterized by quantitative immunofluorescence flow cytometry, using HS and core protein-specific antibodies (see below).

K562 cells were routinely cultured in Dulbecco’s modified Eagle’s medium/2% medium (Life Technologies) supplemented with 10% fetal calf serum. For transfection, 5 × 10<sup>5</sup> cells were incubated with 30 μg of syndecan-1-pREP4, glypican-1-pREP4, or pREP4 plasmid in 0.5 ml of calcium/magnesium-free PBS at 10 min for 4 °C. Cells were then electrotransfected at 250 V and 960 microfarads. Selection with hygromycin B (Roche Molecular Biochemicals) at 200 μg/ml resulted in stable cell populations that were not further subcloned. The population was characterized by quantitative immunofluorescence flow cytometry, using HS and core protein-specific antibodies. Transfections with pREP4 vectors encoding one-chain (SAA), two-chain (SSA), and wild type (SSS) forms of glypican-1 into Namalwa cells were performed in similar ways.

Immunofluorescence Cytometry—Namalwa cells or K562 cells were incubated with 5 μg/ml 10E4 (monoclonal antibody (mAb) recognizing HS) or the core protein-specific antibody BB4 (mAb recognizing syndecan-1, a kind gift from Oxford, United Kingdom) or glypican-1. After 1 h at 4 °C, the cells were washed with PBS plus 2% bovine serum albumin (BSA) and incubated for another 1 h at 4 °C with fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Nordic Immunology, Tilburg, The Netherlands). Cells were washed again with PBS plus 2% BSA and fixed with formaldehyde. The fluorescence was measured with a FACSsort (Becton Dickinson, Mountain View, CA). The value obtained for cells that were incubated with fluorescein isothiocyanate-labeled goat anti-mouse antibodies only was taken as background fluorescence.

Western Blotting—Proteins were extracted from cells with lysis buffer (0.5% Triton X-100 in Tris-buffered saline, supplemented with 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μm sodium-4-nitrophenyl-2′-deoxy-β-D-ribofuranoside) at 4 °C overnight. After extensive washing with lysis buffer and PBS, the beads were resuspended in lysis buffer containing sodium dodecyl sulfate (0.1% N-glycosidase D, 1 unit of N-glycosidase F, Roche Molecular Biochemicals), or only assay buffer at 37 °C overnight. Then the protein A beads were boiled in 2 × SDS sample buffer in the presence of 10 m dithiothreitol. Samples were fractionated by SDS-PAGE and transferred to PVDF membranes. mAb 3F10 was used to detect the HA-FGFR1 in Western blotting.

Cell Surface Biotinylation—HA-FGFR1-transfected or sham-transfected BaF3 cells were washed with cold PBS and incubated with 0.5 mg/ml sulfosuccinimidyl-3-(2-iminothiolane)propionic acid (Pierce) at 4 °C for 20 min. Cells were washed and incubated with 0.5 mg/ml sulfosuccinimidyl-3-(2-iminothiolane)propionic acid for another 20 min at 4 °C. After three washes with cold PBS, cells were lysed with lysis buffer. Streptavidin beads were added to the cell lysate to capture all biotinylated proteins. After 2 h of incubation at 4 °C, the streptavidin beads were washed and boiled in 2 × SDS sample buffer in the presence of dithiothreitol. Both the biotinylated and nonbiotinylated fractions were subjected to SDS-PAGE and then transferred to PVDF membranes. mAb 3F10 was used to detect the HA-FGFR1 in Western blotting.

FGF2-induced HA-FGFR1 Autophosphorylation—BaF3 B6 cells were serum-starved in RPMI medium supplemented with 1 mg/ml BSA and 2% inositol containing medium. After 2 days of starvation, 2 ng/ml FGF2 (Roche Molecular Biochemicals) was added to cells in HEPES-buffered medium (RPMI medium, 25 mM HEPES, pH 7.5, 1 mg/ml BSA, 0.1 mM orthovanadate), in the presence or absence of 100 ng/ml heparin (Calbiochem), at 4 °C for 1.5 h. Then the cells were warmed up at 37 °C for 5–30 min and extracted with lysis buffer in the presence of tyrosine phosphatase inhibitor (1 mM orthovanadate). Cell lysates were clarified by centrifugation at 14,000 × g for 15 min. The supernatants were incubated with PY-20 (anti-phosphotyrosine antibody)-conjugated protein A-agarose beads (Santa Cruz Biotechnology) overnight at 4 °C. The beads were washed with lysis buffer and boiled in 2 × SDS sample buffer in the presence of dithiothreitol. Samples were fractionated by SDS-PAGE and then transferred to PVDF membranes. HA-FGFR1 was detected with mAb 3F10.

For testing the effects of membrane-associated HSPGs on FGF2-induced HA-FGFR1 phosphorylation, 3 × 10<sup>5</sup> B6 cells were incubated with 1 × 10<sup>5</sup> HSPG-presenting cells (fetal human lung fibroblasts; syndecan-1-transfected, glypican-1-transfected, or sham-transfected K562 cells; or transfected Namalwa cells) in the presence of FGF2 at 4 °C for 1.5 h. To remove the cell surface HS, cells were treated with 0.01 units/ml heparinase (Seikagaku Corp., Tokyo, Japan) at 37 °C for 40 min. Treated cells were incubated with B6 cells and FGF2 and analyzed as described above.

For analyzing the time course of the effect of a heparinase digestion, cells were incubated with 0.006 units/ml heparinase (Seikagaku) for 2, 5, 10, or 40 min at 37 °C. Cells were washed twice with assay buffer (HEPES-buffered medium) and then mixed with B6 cells in the presence of 2 ng/ml FGF2. The phosphorylation of HA-FGFR1 was assayed as described above.

To test the soluble forms of these HSPGs, HSPG-presenting cells were treated with 100 μg/ml trypsin (Sigma) at 4 °C for 10 min. Trypsin inhibitor (Sigma) was then added to quench the effect of trypsin. Further treatment with 0.006 units/ml heparinase was performed at 37 °C for 40 min. Soluble HS chains or chain clusters prepared from
imunopurified syndecan-1 or glypican-1 (see below) were incubated with B6 cells in the presence of FGF2, and tested as described above.

**Extraction and Purification of Cell Surface HSPG**—Cell surface proteoglycans were extracted from fetal human lung fibroblasts, syndecan-1- or glypican-1-transfected K562 cells, and transfected Namalwa cells as described previously (4, 22). Briefly, cells were labeled with [35S]Sulfate and lysed with a Triton X-100 buffer in the presence of protease inhibitors. The cell extract was then centrifuged and concentrated on a DEAE-Trisacryl M column (Life Technologies). Proteoglycans were immunopurified with specific mAb, immobilized on CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). After further purification by ion exchange chromatography on a RESOURCE Q column (Amersham Pharmacia Biotech) in Triton-urea-Tris buffer, these HSPGs were treated with chondroitin ABC (Seikagaku) (20 milliunits/ml, 3 h at 37 °C). Soluble HS fractions were obtained from these HSPGs by trypsin digestion (60 μg/ml, 30 min at 37 °C) or alkaline treatment (0.5 % KOH, overnight at 4 °C). All treated proteoglycan fractions were repurified on DEAE beads. Heparitinase digestion (0.015 units/ml) was at 37 °C for 3 h.

**RESULTS**

**Heparin- and FGF2-dependent FGFR1 Phosphorylation in BaF3 Cells**—To test the effects of heparin or HSPGs on receptor activation, a low to zero background of endogenous HSPG expression is required. BaF3 cells express no detectable levels of endogenous HSPG. These cells also cannot be induced to express any heparan sulfate upon transfection with relevant core protein cDNAs, which excludes their use for the design of receptor and HSPG co-transfection experiments. However, several reports suggest that cell surface HSPGs can mediate FGF2 transactivation of the FGFR, using receptor-autophosphorylation as assay. BaF3 cells transfected with an HA epitope-tagged human FGFR1 IgII/IIIc isoform were constructed to act as FGFR1-presenting cells. Namalwa and K562 cells, transfected with syndecan-1 or glypican-1 cDNAs, and fetal human lung fibroblasts, which express high levels of endogenous syndecans and glypican-1, were chosen to act as HSPG-presenting cells.

The expression of HA-FGFR1 in the BaF3 cells was confirmed by Western blotting. HA-FGFR1 appeared as two bands migrating around 100 and 120 kDa. The predicted molecular mass of this HA-FGFR1 is 81.5 kDa, and glycosidase susceptibility tests indicated that the 120-kDa (endoglycosidase H-resistant) and 100-kDa (endoglycosidase H-susceptible) forms represented two different isoforms of glycosylated receptor (both N-glycosidase F-susceptible) (Fig. 1A). Cell surface biotinylation revealed that the majority of the cell surface-exposed HA-FGFR consisted of the 120-kDa isoform (Fig. 1B). HA-FGFR1-transfected BaF3 cells were cloned, and one of the highest expressors, clone B6, was chosen for further experiments. Clone B6 and sham-transfected BaF3 cells were then incubated with or without FGF2 in the presence or absence of heparin, and the phosphorylation of HA-FGFR1 was analyzed as described under “Experimental Procedures” (anti-Tyr(P) pull-down; blotting with anti-HA). Clearly, the phosphorylation (pull-down) of HA-FGFR1 in BaF3 cells was strictly dependent upon the addition of both FGF2 and heparin (Fig. 2A). A time course experiment showed that FGFR1 autophosphorylation reached peak levels around 15 min of exposure to ligand at 37 °C (Fig. 2B).

**Effect of Membrane-associated HSPG on FGF2-induced HA-FGFR1 Phosphorylation**—Cultured human lung fibroblasts accumulate large amounts of syndecans, glypican-1, and perlecan on their cell surfaces (22). Namalwa and K562 cells, in contrast, express very low to low levels of endogenous cell surface HSPG, mainly glypican-1 (in Namalwa cells) and syndecans (in K562 cells) (4). Phorbol diester-stimulated K562 cells secrete significant amounts of perlecan into their culture media (24), but under basic conditions only very little of this proteoglycan can be detected on the surfaces of these cells (result not shown). To construct cells that express only or primarily a single major form of proteoglycan, both the Namalwa and K562 cell lines were transfected with syndecan-1 or glypican-1 cDNA. The expression levels of cell surface syndecan-1 and glypican-1 glycoproteins were monitored by quantitative immunofluorescence flow cytometry, using the HS-specific mAb 10E4 and protein-specific antibodies. In all HSPG-transfected cells, the expression of cell surface HS was markedly increased, by at least 1 order of magnitude. The protein-specific antibodies BB4 and S1 confirmed the expression of cell surface syndecan-1 and glypican-1, respectively (Fig. 3).
HA-FGFR1-transfected BaF3 cells (clone B6) were then incubated with HSPG-presenting cells in the presence of FGF2 and assessed for autophosphorylation of HA-FGFR1. Fig. 4 shows that, independently of their origin, all membrane-associated HSPGs (transfectant syndecan-1 and glypican-1 in the case of Namalwa or K562 cells and total cell surface HSPGs in the case of fetal human lung fibroblasts) markedly enhanced the FGF2-dependent phosphorylation of HA-FGFR1. In contrast, K562 or Namalwa cells that had been treated with heparitinase or transfected with empty vectors had no significant effects on FGFR1 autophosphorylation. This confirmed that the effects of these HSPG-presenting cells on FGFR1 phosphorylation were HSPG-dependent. Interestingly, treating the HSPG-presenting cells with heparitinase instead of heparinase, for increasing lengths of time, first increased and then decreased the capacity of these cells to support FGF2-induced receptor autophosphorylation (Fig. 5).

Effect of Soluble HSPG on FGF2-induced HA-FGFR1 Phosphorylation—Previous reports have demonstrated that purified syndecans and glypican-1 originating from cultured fetal human lung fibroblasts could not assist FGF2 in high affinity receptor binding (11). Nevertheless, in the present assay, cell-associated forms of these HSPGs were able to promote FGF2-induced receptor phosphorylation. To test whether this membrane association played a significant role, the HSPG-presenting cells were treated with trypsin. Trypsin would be predicted to cleave the core proteins of these proteoglycans and to release the ectodomain and ectodomain fragments that bear the HS chains into medium. Analysis of the residual HSPG expression by quantitative immunofluorescence flow cytometry confirmed that after trypsinization, barely any HSPG remained on the cell surface (data not shown). The supernatants of these trypsin digestions (containing the released HSPG) were supplemented with trypsin inhibitor and then added to the HA-FGFR1-transfected B6 cells in the presence of FGF2. The soluble forms of these HSPGs all failed to enhance FGF2-induced HA-FGFR1 phosphorylation (Fig. 4). To ensure that no HS had been lost during the collection of the supernatants, trypsinized HSPG-presenting cells and supernatants were also left together, supplemented with trypsin inhibitor, and added to the B6 cells. None of these recombinations stimulated FGF2-induced receptor phosphorylation (data not shown). Furthermore, when non-trypsin-treated HSPG-presenting cells were mixed with soluble HS, obtained by trypsinizing the equivalent of 10 times more cells, no significant phosphorylation of HA-FGFR1 could be detected in the presence of FGF2 (Fig. 6). Thus, soluble forms of HSPG inhibited the stimulatory effects of cell surface-associated HSPGs.

When cells are treated with trypsin, the core proteins of the proteoglycans (PGs) are at least partially destroyed. To test whether the protein part of the PGs might play a role in assisting FGF2-induced FGFR1 autophosphorylation, glypican-1-transfected Namalwa cells were treated with phosphatidylinositol-specific phospholipase C. This treatment cleaves glycosylphosphatidylinositol tails, leaving the ectodomain of this PG intact. Nevertheless, like trypsin-released HSPG fragments, phosphatidylinositol-specific phospholipase C-released ectodomains of glypican-1 failed to promote FGF2-induced HA-FGFR1 phosphorylation (data not shown).

Since heparitinase can convert inactive HS fractions into activators of FGF2-induced mitogenesis (25), and because a brief heparitinase treatment of the presenting cells stimulated the activity of these cells (see above; Fig. 5), we further treated the trypsin-released HSPGs with heparitinase. Consistently, heparitinase-treated HSPGs stimulated FGF2-induced HA-FGFR1 phosphorylation in all the cases (Fig. 4). In these cases, however, extending the heparitinase-treatment never suppressed the activity of the digest (results not shown). Importantly, adding increasing concentrations of soluble, undigested HS fractions to these heparitinase digests inhibited the stimulatory effect of these digests on HA-FGFR1 phosphorylation (Fig. 6).

2 Z. Zhang, C. Coomans, and G. David, unpublished data.
can-1-transfected Namalwa cells were treated with heparitinase at
harasin-digested trypsin-released soluble HSPG. Activation by FGF only
heparinase-treated cells; HLF, fetal human lung fibroblasts; can-1-transfected K562 cells;
K562-pRep4, digested cells were washed with assay buffer and mixed with aliquots of
(for figure 5) and by the combination of FGF plus heparin (FH) is given as a reference.

Membrane-associated HSPGs of all three cell lines 

Structural Characteristics of HSPGs Purified from Fetal Human Lung Fibroblasts, K562 Cells, and Namalwa Cells—Thus,

HSPGs from three different sources were acting similarly in terms of FGF2-FGFR1 signaling. To assess whether this also reflected similarity in HS structure, syndecan-1 and glypic-an-1 were isolated from detergent extracts of 35S-labeled fetal human lung fibroblasts, K562 cells, and Namalwa cells.

After prepurification on DEAE and affinity purification on corresponding protein-specific antibody columns, the purified PGs were fractionated by ion exchange chromatography over RESOURCE Q. HSPGs derived from human lung fibroblasts and Namalwa cells had fairly homogeneous compositions. Syndecan-1 and glypic-an-1 from human lung fibroblasts were eluted from RESOURCE Q as single sharp peaks (syndecan-1 at 0.58–0.70 M NaCl; glypic-an-1 at 0.56–0.67 M NaCl). Synde-can-1 and glypic-an-1 from Namalwa cells were also eluted as single but slightly broader peaks (at 0.60–0.82 M and 0.48–0.74 M NaCl, respectively). The immunopurified HSPGs from K562 cells, in contrast, were more heterogeneous. Both synde-can-1 and glypic-an-1 eluted as three incompletely separated peaks, with increasing charge densities from peak 1 to peak 3 (Fig. 7) (4). Accordingly, these materials were collected separately, as three distinct subfractions.

The purified and fractionated HSPGs were further digested with chondroitinase ABC and then treated with trypsin, to retain the clustering of the HS chains as in the intact proteoglycan, or with alkali to generate single protein-free HS chains. The fractionation of the single chains from different HSPGs by SDS-PAGE and autoradiography revealed that human lung fibroblasts produced the longest HS chains among the three cell lines (mass of 45–100 kDa for syndecan-1-HS and 40–80 kDa for glypic-an-1-HS). Namalwa (syndecan-1-HS, 18–30 kDa; glypic-an-1-HS, 18–40 kDa) and K562 cells (14–35 kDa) produced shorter chains. Further analysis indicated that the heterogeneity of the K562 materials was due to the differences in both HS chain clustering (one-, two-, and three-chain forms of HSPG) and charge density (data not shown). Altogether, we could deduce from these data that, on average, K562 HS chains derived from peak 3 were more highly sulfated than the HS chains from peak 1 or HS chains from Namalwa or human lung fibroblast HSPGs.

Effects of Purified HSPGs on FGF2-induced HA-FGFR1 Phosphorylation—When trypsin is used to release HS from cells in culture, a mixture of HS chains, HS chain clusters, and degraded cell surface proteins is released into the conditioned medium. To avoid possible interference by these other degraded proteins, single HS chains and chain clusters derived from affinity-purified HSPGs, isolated from fetal human lung
fibroblasts, K562 cells, or Namalwa cells, were also tested in the FGF2-induced HA-FGFR1 phosphorylation assay.

HS fractions derived from fetal human lung fibroblasts or Namalwa cells similarly failed to promote FGF2-induced FGFR1 phosphorylation. Again, after heparitinase treatment all these fractions inhibited the stimulatory effect of the more highly sulfated HS, while the heparitinase digest of all of these HS chains and chain clusters are unable to enhance FGF2-induced FGFR1 phosphorylation, while the heparitinase digests of all of these materials are activators of receptor signaling. Hept, heparitinase. A1, A2, and A3, single HS chains, from peaks 1, 2, and 3; A1 + A2 + A3, pooled peak fractions; T1 and T2, HS chain clusters, from peaks 1 and 2. See Fig. 8 for peak definitions. Other symbols are as in Fig. 4.

**DISCUSSION**

Since Yayon et al. (3) first demonstrated the importance of HSPG for high affinity FGF2-FGFR1 binding, a large amount of biochemical and biological data has been produced, indicating that HSPG is essential for FGF/FGFR signaling (4, 5, 26, 27). Although several models have been proposed from binding studies, functional analyses, and crystal structures (see below), the mechanism through which HSPGs/heparin assist FGF/FGFR signaling remains less well understood. Moreover, the contribution of individual HSPGs to FGFR signaling is also a matter of debate. Two possible reasons for the inconsistency of the published data were addressed here: the cell specificity of the HSPG structure and the importance of membrane association.

Our data indicate that cell surface syndecans and glypican-1 from three different cell lines (fetal human lung fibroblasts,
Namalwa cells were transfected with chain valence mutants of glypican-1. Aliquots of $1 \times 10^6$ B6 cells were incubated with $3 \times 10^6$ cells in the presence of FGF2. The phosphorylation of HA-FGFR1 was analyzed as described under “Experimental Procedures.” Wild type, the two-chain mutant, and the single chain mutant of this glypican all promote FGF2-induced HA-FGFR1 phosphorylation. SSS, wild type; SSA, two-chain mutant; SAA, one-chain mutant. Other symbols as are in Fig. 4.

K562, and Namalwa cells) do differ in fine structure. These structural differences do not appear to play a major role in the activation of FGF2-FGFR1 signaling by membrane-associated HSPGs but correlate with differential activities observed among soluble HSPGs, derived from these membrane forms. Among the soluble HS forms, activity was restricted to highly sulfated subfractions (from K562 cells), the fractions with the highest resemblance to heparin, whose effects on FGF/FGFR signaling are well established. Irrespective of this, the markedly differential effects of soluble and membrane-associated syndecans and glypicans, derived from identical sources and evidently differential effects of soluble and membrane-associated syndecans and glypicans, derived from identical sources and thus with similar glycosylations, suggest that the membrane association of these HSPGs plays an important role for their activity in FGF2/FGFR1 signaling.

It is generally accepted that growth factors activate their cognate receptor tyrosine kinases by inducing receptor dimerization or oligomerization. Various models for FGFR dimerization have been proposed, including a cooperative bridging model (28), a beads on a string model (29, 30), and a preexisting FGFR-HS duplex model (31), to name only some. Recently published crystal structures of a complex between FGF2 and FGFR1 provide a rather convincing receptor dimerization or oligomerization. Various models for FGFR dimerization or oligomerization have been proposed, including a cooperative bridging model (32), except that two HS structures are occupying the canyon while adopting antiparallel end-to-end orientations. The heparin structures bind with their nonreducing ends in the center of the canyon and run out onto the high affinity heparin-binding sites of the ligands. In addition to promoting FGF-FGFR interaction within a 1:1 FGF-FGFR complex, the nonreducing ends of the heparin structures also interact with the adjoining receptor across the 2-fold dimer. In this way, the model provides a molecular basis for the dual role of HS in augmenting 1:1 FGF-FGFR affinity and promoting dimerization of two FGF-FGFR complexes. In the case of intact PGs, this structure would imply the contribution of two heparan sulfate chains, each belonging to a different PG, the protein cores of these PGs (linked to the reducing ends) adopting a peripheral position in the complex. At the moment, it is not clear whether the “two-end” model can be extended to other FGF-FGFR systems. Different FGFs show different HS requirements for the activation of their cognate receptors, and pentameric structures have been reported for FGF1-FGFR2-heparin complexes (35).

 Unlike heparin, heparan sulfate chains have typical block structures and are composed of highly sulfated, heparin-like “S-domains,” alternating with low or nonsulfated domains (36). In the context of the “two-end” model, this would imply that formation of the receptor complex involves two heparan sulfate chains, with relevant S domains as end structures, or very flexible HS chains, with irrelevant end structures that protrude from the canyon. The stimulation resulting from a brief treatment of the cells with heparitinase, interpreted as an increase in the number of heparin-like end structures on cell surfaces, and the activities of the heparitinase-generated fragments of the various soluble HS chains fit well in this “two-end” model. Heparitinase cuts the low sulfated regions of the HS chain but respects the S domains. When heparitinase cleaves cell surface-bound heparan sulfate on the nonreducing side of these S domains, it will convert these domains in end structures. These will remain associated with the cell surface until further cleavage of the chain in the nonsulfated and less sulfated regions, which invariably include the protein-proximal parts of the chains, releases the S domain. The lack of activity of most of the soluble native HS chains and the marked activity of (what we propose to be) the heparitinase-resistant (heparin-like) segments of these HS chains would indicate that most of the relevant S-domains (in terms of receptor dimerization) are embedded in the chains and have only virtual or latent activities. HS chains with appropriate S domains at their nonreducing ends might be sufficiently preponderant among the highly sulfated HS subfractions from K562 cells but would seem to occur less often in other HS fractions tested in this assay. Competition of the internally positioned S domains of these chains for FGF2 binding would easily explain their inhibitory effects on the relatively rare active end structures. In this sense, the effect of heparitinase is to be considered as dually reinforcing, since it both generates active end structures and destroys, by number, the internal inhibitory sequences.

Why then are membrane-associated HSPGs active where their soluble forms fail to activate FGFR signaling? Both forms should have similar ratios of active “end” and inhibitory “internal” structures. Conceivably, direct core protein interactions...
with growth factor and/or receptor could also account for or contribute to receptor activation. The core proteins of some proteoglycans (e.g. NG2 and phosphacan) bind to FGF2 (37, 38). Direct core protein interactions with signaling components might be lost upon tryptic release of the proteoglycan from the cell surface, explaining the failure of these soluble forms. However, at least in the case of glypican-1 this would appear to be insufficient as an explanation, since phosphatidylinositol-specific phospholipase C-released glypican-1 (with an intact ectodomain) also fails to promote FGF2-induced FGFR1 phosphorylation. Possible direct core protein interactions certainly do not suffice for explaining the activity of the membrane-associated forms of syndecan-1 or glypican-1, since this HSPG-assisted FGF2/FGFR1 signaling is strictly HS-dependent. The binding of syndecans and glypicans to FGF2 is solely HS-mediated (4), but interactions with receptor are not excluded. Such interactions would appear to be endowed with little specificity, since syndecan-2 (8) and the membrane-associated HS-substituted splice variant of CD44 (39) also promote FGF2 signaling (mitogenesis) in BaF3 cells. We therefore tend to conclude that membrane association and proteoglycan assemblies rather than a specific direct core protein contribution are needed for efficient HSPG-supported FGF signaling.

It is likely that the concentration of the reactants and the need for threshold concentrations to be reached play a significant role. Cell surface HSPGs binding of FGF will reduce the dimensionality of ligand diffusion from three to two dimensions. Thus, the local concentration of the bound ligands at the plasma membrane and the probability of their interaction with the high affinity receptors might be greatly enhanced. In the present experimental setup, cell binding and cell-cell contacts between HS-presenting cells and receptor-bearing cells are likely to play an important role, which is suggested by the inhibitory effects of adding soluble HSPG to the presenting cells (see above) and adding increasing numbers of presenting cells for fixed amounts of receptor-bearing cells and added FGF (results not shown). Possibly, additional aspects have to be considered. Membrane association of the HSPG might further stabilize the FGF-FGFR-HSPG complex. This might not be essential, since soluble heparitinase-resistant fragments are active in assisting FGF2/FGFR1 signaling, but might occur, since in further studies we were unable to demonstrate “high affinity” binding of FGF2 to cell surfaces supported by such fragments. In contrast, high affinity binding of FGF2 could be measured when FGF2 was presented by cell-bound HSPGs or by heparin, which itself may tether to cell surfaces (40).

Further possible aspects of the membrane association of HSPG, which are not exclusive with the above, include facilitated diffusion and cooperativity. Due to their highly negative charges, HS chains would be expected to repel each other. On most cells, the abundance and molecular dimensions of cell surface HSPGs are such that, assuming a random distribution, they should cover the entire cell surface and even invade each other’s domains. Typical concentrations of HSPGs on the cell surface are in the range of 10^5 to 10^6 molecules/cell (41). Fetal human lung fibroblasts and the HSPG-transfected K562 and Namalwa cells studied here express around 5 x 10^5 to 2 x 10^6 HSPG molecules/cell. According to their molecular masses, the average lengths of the HS chains would vary from 30 to 60 nm in Namalwa and K562 cells, and from 70 to 140 nm in fetal human lung fibroblasts. Around 5 x 10^5 HSPGs uniformly placed on fetal human lung fibroblasts (diameter of 16 μm), K562 cells (14 μm), or Namalwa cells (12 μm), assuming spherical cell shapes, would encompass the entire cell surface. High densities of HS may facilitate FGF diffusion from chain to chain and along chains (42). High density and cell surface association may impose particular chain orientations (all reducing ends oriented toward cells), so that highly sulfated domains at the nonreducing ends of HS chains have better chances to approximate each other than when free in solution. Moreover, when FGFs are added, they will neutralize the negative charges on some chains, reducing the repelling forces between chains. In a simplified model, consisting of two PGs, each with three chains but only one loaded with FGF, fixed in position but with rotational freedom, one would easily conceive how the neutralized chains will be driven together assisted by the repulsion of the nonneutralized chains. This would be more plausible if the neutralized domains were end structures and would result in the FGFs forming a “trans-dimer,” supported by two antiparallel oriented chains.

Glypicans can associate with rafts, resulting in their oligomerization (43). Syndecan core proteins exhibit a propensity to form noncovalently linked dimers and higher order oligomers (44, 45), and adding ligands such as FGF2 to syndecan-1-expressing cells induces clustering of this syndecan (46). Syndecans bind to a variety of intracellular molecules, and the cytoplasmic domain of syndecan-4 seems to play an important role in cellular responses to FGF2 stimulation (47). Thus, syndecans and glypicans may preexist as clusters on the cell surface or be dimerized/oligomerized in response to extracellular ligand binding or inside out signaling, implying that the local concentrations of reactants may even be underestimated in the above reasonings.

In summary, we showed that several HSPG-presenting cells can stimulate FGF2/FGFR1 signaling in neighboring HS-negative/receptor-positive cells but that “proteolytic” shedding of these HSPGs severely impairs their activity in this sense. This indicates HSPG shedding could have a major role in negatively regulating growth factor activity. The physiological relevance of this experimental paradigm remains to be proven but is plausible, since in vivo the HS expressions are often restricted to particular cells and since inhibitory shedding and/or accumulation in inflammatory fluid (19). Bacterial heparitinase converted these inhibitory shed HS species into activators of FGF2-dependent FGFR1 autophosphorylation, interpreted as the generation of “end structures” that can cooperate in receptor dimerization (34). Mammalian heparanase is an endoglycosidase, cleaving heparan sulfate at specific intrachain sites (48). Whether mammalian heparanases produce “end-structures” of the types that were used in modeling and crystal structures and that we suggest to be generated here remains to be proven. Nevertheless, mammalian heparanases can also convert inhibitory soluble HS PG ectodomains, recovered from inflammatory fluid, into potent mitogenic activators of FGF2 (19). All cells tested here express mammalian heparanase.2 In the extension of this model, the form of HSPG that activates FGF2 signaling might be a heparanase-generated product.

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Membrane Heparan Sulfate Proteoglycan-supported FGF2-FGFR1 Signaling: EVIDENCE IN SUPPORT OF THE "COOPERATIVE END STRUCTURES" MODEL

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J. Biol. Chem. 2001, 276:41921-41929.
do: 10.1074/jbc.M106608200 originally published online September 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106608200

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