Heparan Sulfate Proteoglycans Function as Receptors for Fibroblast Growth Factor-2 Activation of Extracellular Signal–Regulated Kinases 1 and 2

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Abstract—Fibroblast growth factor-2 (FGF2) activates the extracellular signal–regulated kinases 1 and 2 (ERK1/2) through its specific receptors. Interaction of FGF2 with cell-surface heparan sulfate proteoglycans has also been suggested to induce intracellular signals. Thus, we investigated whether FGF2 can stimulate ERK1/2 activation through heparan sulfate proteoglycans using mechanisms that do not depend on receptor activation in vascular smooth muscle cells. The activation of FGF receptors was inhibited by treating cells with 5′-deoxy-5′-methyl-thiadesoxyadenosine and by expressing truncated dominant-negative FGF receptors. In both cases, FGF2 was able to stimulate the phosphorylation of ERK1/2 despite the absence of detectable FGF receptor tyrosine kinase activity. The FGF2 activation of ERK1/2 in the absence of receptor activity was completely dependent on heparan sulfate, because this activity was abolished by heparinase III digestion of the cells. In contrast, heparinase III treatment of control cells, with functional FGF receptors, showed only slight changes in FGF2-mediated ERK1/2 activation kinetics. Thus, in addition to serving as coreceptors for FGF receptor activation, heparan sulfate proteoglycans might also function directly as receptors for FGF2-induced ERK1/2 activation. Activation of ERK1/2 via cell-surface proteoglycans could have significant biological consequences, potentially directing cell response toward growth, migration, or differentiation. (Circ Res. 2004;94:316-323.)

Key Words: fibroblast growth factors ■ growth factors

Fibroblast growth factor-2 (FGF2) is one of the best-characterized members of the FGF family, and it has been demonstrated to be an important modulator of cell growth, differentiation, and migration.1,2 Binding of FGF2 to cellsurface tyrosine kinase FGF receptors (FGFRs) triggers receptor dimerization and autophosphorylation of tyrosine residues, which act as docking sites for downstream proteins leading to cell response.3 FGF2 also binds to heparan sulfate proteoglycans (HSPGs), complex molecules composed of a core protein and covalently attached heparan sulfate chains.4 HSPGs are found in the extracellular matrix and on cell surfaces of most tissues, where they serve many functions.4,5 In the vascular system, HSPG and heparin have been demonstrated to inhibit vascular smooth muscle growth in vitro and in vivo.6–8 Although one mechanism suggests that heparin and heparan sulfate can sequester growth factors, such as FGF2, to prevent cell stimulation,9 others studies have shown that HSPGs are necessary for the maximal mitogenic activity of FGF2.2,4,10 Indeed, when agents were delivered to degrade or decrease HSPG, both enhanced and inhibited smooth muscle cell hyperplasia was observed.11–14 Thus, it is clear that HSPGs play important roles in the blood vessel wall, yet the underlying mechanisms used by HSPG remain poorly understood.

Cell-surface HSPGs have been demonstrated to modulate FGF2 activity by functioning as coreceptors to enhance FGF2 binding to FGFRs.2,10 However, recent studies suggest that the role of HSPG in the FGF2 system might be more complex. Indeed, FGF2 might signal directly via cell-surface HSPG by inducing aggregation of syndecan-4 and activation of protein kinase Ca.15,16 Thus, a more complete understanding of how HSPGs modulate the vascular response to FGF2 is required before approaches can be designed to treat vascular disease by targeting HSPGs.

The syndecans are a widely distributed 4-member family of transmembrane proteins carrying both heparan and chondroitin sulfate chains.4 Although there are significant differences within their ectodomains, the four syndecans share a highly conserved cytoplasmic tail, suggesting important functions.4 Indeed, Horowitz and Simons16,17 showed that the cytoplasmic tail of syndecan-4 is phosphorylated on Ser183 and that FGF2 can cause a 2- to 3-fold reduction in phosphorylation, inducing multimerization and activation of protein kinase Ca. FGF2-mediated signaling via syndecan-4...
might proceed to downstream targets, such as the extracellular signal–regulated kinases 1 and 2 (ERK1/2), to regulate cell activity independent of FGFRs. Activated ERK1/2 regulates cell function by phosphorylating proteins, such as downstream kinases, cytoskeletal elements, regulators of apoptosis, and several transcription factors.18 The biological response mediated by ERK1/2 is dependent on the extent, kinetics, and context of activation. Hence, ERK1/2 could be a site of convergence for FGF2-mediated signaling through HSPG and FGFR.

To study the potential role of HSPG in FGF2-mediated ERK1/2 activation, we carried out experiments in bovine vascular smooth muscle cells (VSMCs), an FGF2-responsive cell type that expresses syndecan-4 and only one of the FGFR isotypes (FGFR1c19). We selectively inhibited FGF2 signaling in VSMCs and showed that FGF2 was still able to activate ERK1/2. Furthermore, the putative FGFR-independent ERK1/2 activation was completely abolished in cells pretreated with heparinase III, a specific heparan sulfate lyase. Thus, in addition to serving as coreceptors for FGF2 activation, HSPGs might also function as receptors for FGF2-induced ERK1/2 activation.

Materials and Methods

Materials

Bovine VSMCs (line AG08502A) were from Coriell Cell Repositories (Camden, NJ). Chinese hamster ovary (CHO) cells (clone ldlD) were obtained from Dr Monty Krieger at MIT (Cambridge, Mass). Cell culture reagents were from Invitrogen. Recombinant human FGF2 was a gift from Chiron, Inc (Emeryville, Calif). Heparinase III (HepIII) was a gift from BioMarin Pharmaceuticals Inc (Montreal, Canada). Mouse anti-myc tag antibodies and rabbit polyclonal antibodies against the asialically phosphorylated Thr/P/Tyr(P) ERK1/2 were from New England Biolabs (Beverly, Mass). Anti-phosphotyrosine antibody, PY20, was purchased from BD Sciences (Lexington, Ky). Rabbit polyclonal anti-ERK1/2 was from Upstate Biotechnology (Lake Placid, NY). 5′-deoxy-5′-methyl-thioadenosine (MTA) and secondary peroxidase-conjugated goat anti-rabbit antibodies were from Sigma (St Louis, Mo). AG1478 was from Calbiochem. pGEM-T cloning vector was from Promega. pCLNX2 viral vector was from Boston University School of Medicine.

Cell Cultures and Treatment

VSMCs (passages 7 through 12) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum (CS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. For experiments, VSMCs were plated in growth media and incubated at 37°C for 72 hours, such that they were ~50% confluent at the time of treatment (~50 000 cells/cm²), with most (90% to 93%) of the cells in G0/G1 and 1% in S-phase. CHO cells were maintained in Ham’s F12 media containing 10% CS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. For experiments, CHO cells were cultured under serum-free conditions in Ham’s F12 containing 1% insulin, transferrin, selenium mixture (Becton Dickinson), and 1 mmol/L galactose. For HepIII treatment, cells were pretreated with HepIII (0.1 U/mL) for 1 hour at 37°C. The pretreatment abolished >98% of FGF2 binding to cell-surface HSPG (data not shown). Cells were then treated with control media (low-glucose DMEM, 0.1% bovine serum albumin [BSA], 25 mmol/L HEPES) with or without HepIII (0.1 U/mL) or MTA (3 mmol/L) for 20 minutes at 37°C. FGF2 was added to the media, and the cells were incubated for the indicated time. Treatment of the cells for 20 minutes with 3 mmol/L MTA before the addition of FGF2 eliminated detectable FGFR signaling (Figure 1A). MTA treatment was fairly specific, because it had little effect on epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor autophosphorylation, as previously reported (Figure 1B).20

Western Blot Analyses

At the end of the treatment, cells were solubilized with lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L EGTA, and 0.5% NP-40) and boiled for 5 minutes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added, and samples were boiled for 3 additional minutes. Samples were subjected to 12% SDS-PAGE and electrottransferred to Immobilon-P membranes. Membranes were blocked in 5% nonfat milk and probed with primary antibody for 1 hour at 37°C followed by 1-hour incubation with horseradish peroxidase–linked secondary antibody at 37°C. Bands were visualized with ECL (Amersham Biosciences Corp). To quantitate ERK1/2 activation, band density (ERK1/2 together) was determined using Scion Image (Scion Corporation), and the density of each sample was normalized by dividing the intensity of ERK1/2 in a lane containing a standard sample of activated VSMC extract (run on every gel to control for exposure). To control for loading, each normalized phospho-ERK1/2 value was divided by its total ERK1/2 value, which was determined by dividing the density of the total ERK1/2 in each sample by the level of total ERK1/2 in the positive control lane (Normalized Activation, Equation 1). FGF2-induced ERK1/2 activation levels were calculated by subtracting the normalized level of activated ERK1/2 in nontreated samples from that for the corresponding FGF2-stimulated samples (Equation 2), as follows:

$$\frac{(\text{Active ERK1/2}/\text{Positive Control Active ERK1/2})}{(\text{Total ERK1/2}/\text{Positive Control Total ERK1/2})} = \text{Normalized Activation} \tag{1}$$

$$\text{Activation Level} = \text{FGF2-Treated Normalized Activation} − \text{Untreated Normalized Activation} \tag{2}$$

Dominant-Negative FGFR1 Mutant

To create a dominant-negative FGFR1, we deleted the entire cytoplasmic domain of FGFR1. The truncated FGFR1 was created using PCR. To this end, the sense primer was designed with a NotI site and extended to include 14 bases of the FGFR1 gene. The anti-sense

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(1) FGF2-Treated Normalized Activation

(2) Activation Level = FGF2-Treated Normalized Activation

− Untreated Normalized Activation
Viral Production
DNA 30 μg and lipofectamine 75 μL in 2 mL DMEM were incubated at room temperature for 45 minutes before adding to the 293GPG cells. Cells were incubated at 37°C for 6 hours, followed by the addition of 4 mL of growing media. Cells were incubated at 37°C for 72 hours before the virus-containing media was collected for 7 days. The virus-containing media mixed with 1 mL of polybrene just before being used to infect 70% confluent VSMCs overnight. The infected VSMCs were trypsinized and grown in media supplemented with G418 (500 μg/mL) for 4 days. At day 4, VSMCs (X² and Δ) were trypsinized and plated for experiments.

125I-FGF2 Cell-Surface Binding
125I-FGF2 was prepared by a modified Bolton-Hunter procedure. Cells were plated at 80% confluence in growing media overnight at 37°C. Cells were washed once with binding buffer (DMEM, 0.1% BSA, 25 mM HEPES) and incubated in binding buffer at 4°C for 15 minutes. 125I-FGF2 was added, and the cells were incubated for 2.5 hours at 4°C. After the incubation, cells were washed three times with ice-cold binding buffer. This was followed by extraction with high-salt (20 mM HEPES, 1 mol/L NaCl, pH 7.4) and low-pH (20 mM sodium acetate, 1 mol/L NaCl, pH 4) buffers to remove HSPG-bound and FGFR-bound 125I-FGF2, respectively. 125I-FGF2 was counted using a γ counter.

[3H]Thymidine Incorporation
VSMCs (X² and Δ) were plated at 5000 cells/cm² in growth media. After 72 hours, cells were pretreated with or without HepIII (0.1 U/mL) for 1 hour, and the media were replaced with binding buffer with or without HepIII (0.1 U/mL) containing [3H]thymidine (1 μCi/mL). FGF2 (0.2 ng/mL) or CS (10%) was added, and at the designated time, the cells were fixed with methanol and precipitated with 5% trichloroacetic acid. [3H]DNA was quantified by scintillation counting.

Cell Migration
VSMCs (X² and Δ) were plated at 5000 cells/cm² into 8.0-μm-pore polycarbonate culture inserts (Nunc) and incubated at 37°C for 72 hours. The cells were subjected to treatment with or without 0.1 U/mL of HepIII for 1 hour, and the media were replaced with binding buffer (±0.1 U/mL HepIII). Binding buffer containing FGF2 (0.5 ng/mL), CS (10%), or no additives was added to the bottom chamber, and the cultures were incubated at 37°C for 24 hours. After the migration period, the media were removed and the membranes were washed once with PBS. Cells on top of the membrane were removed using Q-tip swabs, and the relative number of cells that migrated to the bottom was measured by assaying for acid phosphatase.

Results
FGF2 Differentially Activates ERK1/2 in VSMCs
To evaluate various FGF2-mediated pathways for ERK1/2 activation, VSMCs were treated with FGF2 under conditions designed to limit the kinase activity of the FGFR (MTA-treated) and to degrade cell-associated heparan sulfate (HepIII-treated). FGF2 differentially activated ERK1/2 under the various treatment conditions (Figure 2). Figure 2A shows representative autoradiograms from FGF2-treated and untreated samples. Figures 2B and 2C represent the calculated levels of FGF2-activated ERK1/2 under the various treatment conditions determined by normalizing the band intensities in the samples to an internal control run on each gel and the level of total ERK1/2 in each sample. In control cells, ERK1/2 phosphorylation was detected at 2 minutes and reached a maximum at 15 minutes after FGF2 stimulation. ERK1/2 remained phosphorylated even 4 hours after treatment. When we pretreated cells with HepIII to remove
cell-surface HSPG, we observed a shift in the activation pattern. In HSPG-deficient cells where FGF2 was only able to signal through the cell-surface FGFR, ERK1/2 phosphorylation could be detected as early as 1 minute after treatment and approached peak activation after 5 minutes that remained high at 4 hours after treatment. Interestingly, in cells pretreated with MTA, which prevented FGFR activation, we also observed ERK1/2 phosphorylation after the addition of FGF2. The kinetics were delayed compared with the control and HepIII-treated conditions. ERK1/2 activation was first detected at 15 minutes and persisted for 4 hours (Figures 2A and 2B). The data suggest that FGF2 can activate ERK1/2 through HSPG. Indeed, when MTA-treated cells were also pretreated with HepIII, FGF2-stimulated ERK1/2 activation was eliminated (Figures 2A and 2C).

An analysis of the long-term kinetics of FGF2-mediated ERK1/2 activation was also conducted (Figure 3). For the control and HepIII- and MTA-treated conditions, the level of ERK1/2 activation decreased over this time course. However, activation was somewhat more persistent in both the HepIII- and MTA-treated cells compared with control. Cells treated with both HepIII and MTA showed nearly undetectable levels of activated ERK1/2 at all time points. These data indicate that the absence of one pathway (HSPG degradation or receptor inhibition) does not eliminate FGF2-mediated activation of ERK1/2, suggesting that HSPG and FGFR can signal independent of one another in response to FGF2 under certain conditions. However, the differences in the profiles of activation in the control condition compared with HepIII or MTA treatment alone suggest that the FGF2-HSPG-FGFR ternary complex signals differently than either of the component binary complexes (FGF2-HSPG or FGF2-FGFR).

Not All HS-Binding Growth Factors Activate ERK1/2 Through HSPG

To examine whether other heparin-binding growth factors can activate ERK1/2 through HSPG, we compared the activation of ERK1/2 by FGF1, FGF2, FGF10, EGF, and HB-EGF. VSMCs were pretreated with HepIII to digest heparan sulfate, MTA to inhibit FGFR, or AG1478 to block EGFR signaling (Figure 4). All of these growth factors except EGF have been shown to bind HSPG. FGF1, like FGF2, activated ERK1/2 in cells treated with MTA, suggesting that FGF1 can activate ERK1/2 through an FGFR-independent manner. Furthermore, ERK1/2 phosphorylation was reduced significantly when both FGFR and HSPG signaling were blocked by combined treatment with MTA and HepIII. In contrast, FGF10 did not activate ERK1/2 when FGFR was inhibited (MTA treatment). FGF10 stimulated ERK1/2 in the control condition, consistent with the ability to activate FGFR1. Activity was decreased when cells were pretreated with HepIII, presumably because of weaker interaction between FGF10 and FGFR in the absence of cell-surface HSPG. Similarly, activation of ERK1/2 by EGF and HB-EGF was not significantly dependent on HSPG. Both growth factors showed time-dependent ERK1/2 activation, which was abolished when the cells were pretreated with AG1478, a potent EGF receptor inhibitor, but not significantly affected when the cells were pretreated with HepIII.

Dominant-Negative FGFR1

To additionally investigate possible FGFR-independent pathways for FGF2 activation of ERK1/2, we created VSMC
mutant cells that overexpress a dominant-negative form of FGFR1. We designed DNA primers to flank the entire extracellular domain, transmembrane domain, and the first 64 amino acids of the intracellular domain of FGFR1. This mutant was designed to retain normal extracellular activity but lack the ability to phosphorylate or be phosphorylated. The dominant-negative FGFR1 was expressed in the mutant cells (VSMC-\(\Delta\)/H9004), as determined by immunoblot using an anti-myc antibody to detect the myc tag on the mutant protein (Figure 5A). Furthermore, when FGF2-stimulated VSMC-X2 and VSMC-\(\Delta\)/H9004 were analyzed for FGFR tyrosine kinase activity by immunoblot with Tyr-P antibody (PY20), significant FGF2-mediated phosphorylation of the major 90-kDa band was only observed in VSMC-X2 treated with FGF2, FRS2 phosphorylation was undetectable in VSMC-\(\Delta\) treated with FGF2. C, Binding of \(^{125}\)I-FGF-2 (100 ng/mL) to confluent SMC-wt (filled), VSMC-X2 (open), and VSMC-\(\Delta\) (shaded) revealed increased receptor expression in the VSMC-\(\Delta\). Binding of \(^{125}\)I-FGF-2 to HSPG and receptor sites was determined using selective extraction with high-salt and low-pH buffers (see Materials and Methods). Data are the averages of triplicates (=SEM).

Figure 5. Characterization of VSMC-\(\Delta\). A, SMC-wt, VSMC-X2, and VSMC-\(\Delta\) whole-cell lysates were resolved by gel electrophoresis, and membranes were probed with anti-myc antibody. Only VSMC-\(\Delta\) containing the myc-tagged FGFR1 showed a positive signal. B, VSMC-X2 and VSMC-\(\Delta\) were stimulated with FGF2 (1 ng/mL) for 5 minutes. Whole-cell lysates were probed with PY20 antibody. Whereas FRS2 (arrow) was phosphorylated when VSMC-X2 was stimulated with FGF2, FRS2 phosphorylation was undetectable in VSMC-\(\Delta\) treated with FGF2. C, Binding of \(^{125}\)I-FGF-2 (100 ng/mL) to confluent SMC-wt (filled), VSMC-X2 (open), and VSMC-\(\Delta\) (shaded) revealed increased receptor expression in the VSMC-\(\Delta\). Binding of \(^{125}\)I-FGF-2 to HSPG and receptor sites was determined using selective extraction with high-salt and low-pH buffers (see Materials and Methods). Data are the averages of triplicates (=SEM).

ERK Activation in VSMC-\(\Delta\) Is Abolished by HepIII

VSMC-\(\Delta\) were treated with FGF2, and ERK1/2 activation was detected, with a peak observed between 5 and 30 minutes, followed by a gradual decline toward basal level by 4 hours (Figure 6A). When VSMC-\(\Delta\) were pretreated with MTA, FGF2 stimulated ERK1/2 activation to a similar extent, with a peak at 15 minutes and a decline toward baseline by 4 hours. Thus, FGF2 was able to stimulate ERK1/2 phosphorylation in the absence of functioning FGFR kinase in VSMC-\(\Delta\). If the FGF2-mediated ERK1/2 activation observed in VSMC-\(\Delta\) was the result of HSPG signaling, then this activity should be eliminated by HepIII treatment. Indeed, when VSMC-\(\Delta\) were pretreated with HepIII before FGF2 addition, ERK1/2 phosphorylation was barely detectable (Figure 6B). The dramatic effect of HepIII on VSMC-\(\Delta\)
is in contrast to that observed with wild-type cells, where HepIII treatment alone did not significantly reduce the level of ERK1/2 activation. The data are consistent with a mechanism where FGF2 is able to stimulate ERK1/2 activation through cell-surface HSPG independent of active FGFR receptors. It is interesting to note that the ERK1/2 activation profile in the VSMC-Δ cells is more transient than that observed with the receptor-inhibited wild-type cells (MTA-treated). This possibly reflects the decrease in HSPG available for FGF2 binding on the VSMC-Δ cells compared with the wild-type cells. However, in both instances (VSMC-Δ and MTA-treated wild-type cells), HepIII treatment resulted in nearly complete loss of FGF2-mediated activation, suggesting that the mechanism for ERK1/2 activation is similar in these two situations. As additional evidence that FGF2 can activate ERK1/2 in the absence of functional FGFR, we also observed FGF2 activation of ERK1/2 in CHO cells that contain undetectable FGFR levels. FGF2 activated ERK1/2 in CHO cells after 15 minutes of treatment with 0.5 ng/mL (activation level, 0.65 U), whereas MTA treatment caused only a slight reduction (activation level, 0.58 U). HepIII treatment reduced FGF2 activation of ERK1/2 by nearly 50% (activation level, 0.35 U), and HepIII treatment in the presence of MTA resulted in a complete loss in FGF2 stimulation of ERK1/2 in CHO cells (activation level, 0.04 U).

Activation of ERK1/2 by FGF2 has been demonstrated to be involved in most FGF2-mediated biological responses. Thus, to determine whether the receptor-independent FGF2 activation of ERK1/2 can lead to cellular response, we analyzed FGF2 stimulation of DNA synthesis and cell migration in VSMC-Δ and VSMC-X (Figure 7). Quiescent VSMC-Δ and VSMC-X were stimulated with FGF2 for various periods, and [³H]-thymidine incorporation into DNA was measured. FGF2 treatment stimulated DNA synthesis in the parent VSMC-X (Figure 7A). HepIII treatment caused a slight reduction in FGF2-stimulated DNA synthesis (≈35% decrease). VSMC-Δ, in contrast, showed no significant stimulation by FGF2 in the presence of HepIII over the entire time course, suggesting that FGFR signaling is required for FGF2-mediated mitogenesis. To ensure that the differences in FGF2-induced mitogenesis observed between VSMC-Δ and VSMC-X did not reflect a generalized difference in the mitogenic activity of the cell lines, we also compared the response to CS (Figure 7B). CS stimulated DNA synthesis in VSMC-Δ and VSMC-X to a similar extent, both in the absence and presence of HepIII treatment. Therefore, putative HSPG-mediated signaling does not seem to be sufficient or absolutely required for FGF2 to induce VSMC proliferation.

The ability of FGF2 to stimulate VSMC migration also seems to require functional FGFR, because FGF2 was unable to stimulate VSMCΔ migration. However, unlike with the mitogenic response, FGF2 stimulation of cell migration was completely dependent on HSPG, because HepIII treatment of VSMC-X eliminated the response to FGF2 (Figure 7C). Although the lack of FGF2 stimulation of VSMC-Δ migration indicates that HSPG signaling is not sufficient for this activity, the requirement for HSPG in VSMC-X migration suggest an important function for HSPG in this process. These data suggest that both FGFR- and HSPG-mediated signaling are required for FGF2 to induce cell migration; alternatively, the requirement for HSPG might solely be a consequence of the ability of HSPG to serve as coreceptors to enhance FGFR signaling. In any case, the inability of FGF2 to stimulate cell growth or migration in the VSMC-Δ is.

Figure 7. FGF2 stimulation of DNA synthesis and cell migration. A and B, DNA synthesis was measured after the indicated period of treatment with FGF2 (0.2 ng/mL, A) or CS (10%, B) in VSMC-XΔ (● and ⋄) and VSMC-Δ (○ and ▲) in the absence (○ and ▲) or presence (● and ⋄) of HepIII. Data represent the average ³H incorporation (cpm) in triplicate cultures ± SEM. C, Cell migration in response to FGF2, HepIII, or CS was measured after 24 hours for VSMC-XΔ (filled bars) and VSMC-Δ (shaded bars). Data are the averages of triplicates ± SEM of acid phosphatase activity (A400 nm).
additional evidence that the level of expression of the dominant-negative FGFR was sufficient to block FGFR function in these cells.

**Discussion**

HSPGs have been implicated in biological functions of a wide range of heparin-binding growth factors, including members of the FGF family.\(^2,4,26\) Whereas the regulation of growth factor activity by HSPG has been attributed to the ability to regulate growth factor–receptor interactions, recent evidence suggests that HSPG can directly participate in signaling.\(^2,4\) In the present study, we showed that HSPGs mediate FGF2 activation of ERK1/2 in the absence of functional FGFR. Thus, in addition to participating in regulating FGFR activity, these data suggest that HSPG can also serve directly as receptors for FGF2.

We prevented the activation of FGF receptors on VSMCs by treating cells with MTA and by expressing truncated dominant-negative FGFR receptors, yet FGF2 was able to stimulate the phosphorylation of ERK1/2. The FGF2 activation of ERK1/2 in the absence of FGFR activity was completely dependent on HSPG, because this activity was abolished by HepIII digestion of heparan sulfate on VSMCs. Interestingly, HepIII treatment of control cells did not dramatically alter FGFR-mediated ERK1/2 activation in VSMCs. This is somewhat in contrast with the known coreceptor role of HSPG for FGF2 binding and activation of FGFR signaling that has been described in other systems.\(^2,4,26\) Although FGF-mediated ERK1/2 activation kinetics in our VSMC system are not altered significantly by HepIII treatment, we observed that HepIII treatment prevented FGF2 from stimulating VSMC migration and caused a partial decrease in FGF2-induced mitogenesis (Figure 7).\(^25\) In addition, CHO cells that express significantly less FGFRs than VSMCs showed a greater dependence on HSPG, because HepIII treatment alone caused a significant reduction in FGF2-mediated ERK1/2 activation (~50%), suggesting that level of FGFR expression may impact the observed dependence on HSPGs as coreceptors. Hence, the activation of ERK1/2 by FGF2 is sensitive to many factors and is not, alone, an accurate predictor of biological response. Instead, ERK1/2 activation likely represents one important component of a complex array of factors that work in concert to discriminate between the myriad of FGF2-mediated biological activities.

The ability of FGF2 to activate ERK1/2 through HSPG in an FGFR-independent manner suggests that HSPGs might function as generalized receptors for a wide range of heparin-binding proteins. Indeed, several studies have identified receptor-like functions for syndecan-3 and -4. Syndecan-3 has been shown to act as a receptor for heparin-binding growth-associated molecule to mediate neurite outgrowth via src signaling,\(^29\) whereas syndecan-4 has been demonstrated to function as a receptor for the multidomain protein, a disintegrin and metalloprotease 12, to mediate cell spreading through protein kinase Ca and RhoA.\(^30\) Moreover, several studies have implicated syndecan-4 clustering and protein kinase Ca activation in regulating cell adhesion, migration, and proliferation.\(^27,31,32\)

Although the mechanisms by which FGF2 stimulates syndecan-4 clustering and signaling are not known, it has been suggested that FGF2 induces a redistribution of syndecan-4 to cholesterol-rich lipid rafts, where access to cytoplasmic signaling molecules may be enhanced.\(^33\) Clustering of syndecan-4 might be mediated by the self-association of FGF2 molecules bound to heparan sulfate chains on separate syndecan-4 molecules. Heparin and heparan sulfate have been shown to enhance the formation of noncovalent FGF2 dimers and higher-order oligomers in a side-to-side orientation.\(^34\) Alternatively, the binding of FGF2 to HSPG could facilitate the interaction of the heparan sulfate chains with heparin receptors on the cell, which have been demonstrated to lead to ERK1/2 activation in VSMCs.\(^35\)

It is interesting that not all of the heparin-binding growth factors that were analyzed activated ERK1/2 through HSPG. Whereas FGF1 and FGF2 showed receptor-independent and heparan sulfate–dependent activation of ERK1/2, FGF10 and HB-EGF were unable to activate ERK1/2 when their respective receptors were inhibited. These growth factors show differences in their ability to bind to heparin, suggesting that activation of HSPG signaling might depend on the specific heparan sulfate sequence that the growth factor binds to and may be controlled by the physical positioning and density of growth factor–binding sites on the heparan sulfate chains of the HSPG.\(^26,36\) Thus, the existence of an HSPG-mediated pathway for ERK1/2 activation might provide additional levels of control over growth factor regulation of cell function.

ERK1/2 activation is a key point of convergence for many signaling pathways, and the biological response of many extracellular stimuli has been demonstrated to require ERK1/2 activation. However, the particular biological response that is mediated by ERK1/2 depends on the context in which it is activated.\(^38\) Thus, the ability of HSPG to participate in ERK1/2 signaling adds increasing evidence for the importance of HSPGs as active regulators of cell function. A complete understanding of the function of heparin-binding growth factors will need to include an appreciation of possible receptor-independent activities mediated by HSPG.

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**References**


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