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Spatial and temporal expression of heparan sulfate in mouse development regulates FGF and FGF receptor assembly

Benjamin L. Allen and Alan C. Rapraeger

Program in Cellular and Molecular Biology, Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53706

Heparan sulfate (HS) interacts with diverse growth factors, including Wnt, Hh, BMP, VEGF, EGF, and FGF family members, and is a necessary component for their signaling. These proteins regulate multiple cellular processes that are critical during development. However, a major question is whether developmental changes occur in HS that regulate the activity of these factors. Using a ligand and carbohydrate engagement assay, and focusing on FGF1 and FGF8b interactions with FGF receptor (FR)2c and FR3c, this paper reveals global changes in HS expression in mouse embryos during development that regulate FGF and FR complex assembly. Furthermore, distinct HS requirements are identified for both complex formation and signaling for each FGF and FR pair. Overall, these results suggest that changes in HS act as critical temporal regulators of growth factor and morphogen signaling during embryogenesis.

Introduction

Heparan sulfate (HS) is a sulfated glycosaminoglycan expressed on core proteins at the cell surface and in the ECM (Bernfield et al., 1999). HS modulates the activity of multiple growth factor families, including FGFs, EGFs, VEGFs, and HGF (Esko and Selleck, 2002). Similarly, HS also regulates the activities of morphogens, including Wnts, Hh, and BMPs (Nybakken and Perrimon, 2002). Because these morphogens and growth factors are critical for embryogenesis, an important question is whether developmental changes in HS regulate the activities of these proteins. This is difficult to answer due to technical problems with obtaining and analyzing HS from discrete sites at specific developmental stages. Currently, only gross analyses of overall HS sulfation levels in whole tissues are possible (Safaiyan et al., 2000). These approaches fail to identify the subtle differences in HS sulfation patterns thought to regulate HS-binding proteins. Conversely, HS-specific antibodies identify different HS epitopes (Dennissen et al., 2002), but fail to demonstrate the functionality of these differences with regard to growth factor and morphogen signaling.

In this paper, a ligand and carbohydrate engagement (LACE) assay was used to map the ability of exogenous growth factors and soluble growth factor receptors to interact with HS in situ at different stages of embryogenesis. FGFs were chosen for this analysis for several reasons. First, the 22 known FGFs regulate a multitude of developmental processes including development of the limb, lung, heart, and brain (Goldfarb, 1996). Second, although HS regulates the activities of a number of different morphogens, this ability is best understood for the FGFs (Ornitz, 2000). Studies using cells deficient in HS first demonstrated a requirement of HS in the formation of a high affinity FGF and FGF receptor (FR) complex (Yayon et al., 1991), whereas studies using chemical inhibitors of HS synthesis demonstrated a requirement for HS in FGF signaling (Rapraeger et al., 1991). Third, studies have shown that the requirement for HS represents an interaction between not only FGFs and HS (Faham et al., 1996) but also between FRs and HS (Kan et al., 1999). Finally, previous work has identified tissue-specific differences in HS that regulate the ability of both FGF and FGF–FR complexes to interact with HS (Allen et al., 2001).

Because HS has been shown to undergo changes during development (David et al., 1992; Jenniskens et al., 2002), the goal of this paper is to determine whether such developmental changes in HS might regulate morphogen signaling. To

Address correspondence to Alan C. Rapraeger, Dept. of Pathology and Laboratory Medicine, 1300 University Ave., University of Wisconsin, Madison, WI 53706. Tel.: (608) 262-7577. Fax: (608) 265-3301. email: acraprae@wisc.edu

Key words: fibroblast growth factors; FGF receptors; development; proteoglycan

Abbreviations used in this paper: 2-ODS, 2-*O*-desulfated; FR, FGF receptor; FRAP, FR–alkaline phosphatase; HAB, heparin-agarose bead; HBD, heparin-binding domain; HS, heparan sulfate; HS2ST, HS 2-*O*-sulfotransferase; LACE, ligand and carbohydrate engagement.

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address this, LACE was performed using two FGFs: FGF1 and FGF8b, and two FRs: FR2c and FR3c. These FGFs and FRs interact with one another, thus providing four different pairs of probes for analysis. In addition, these FGFs and FRs have been implicated in a number of developmental processes, including heart formation (FGF1 and FGF8b), limb development (FGF8b), and bone growth (FR2c and FR3c).

This paper identifies dramatic differences in the in situ interactions of these four FGF-FR pairs with HS at different developmental stages. In fact, for each FGF-FR pair examined, unique developmental HS binding patterns are identified that correlate with different HS binding requirements, as well as differences in FGF signaling. These results suggest that each FGF-FR combination seeks distinct HS domains that are spatially and temporally regulated during development. These domains are unique to HS and distinguish it from heparin, which is highly sulfated and lacks any domain structure, thus explaining the ability of these FGF-FR pairs to interact equally with heparin in vitro. Importantly, the HS activity necessary for ternary complex assembly does not represent the additive binding requirements of individual FGFs or FRs; rather, it represents requirements dictated by the synergistic interaction of the FGFs, HS, and FRs. Finally, given that HS uniquely mediates each FGF–FR interaction examined here, these results suggest that developmental changes in HS may also specifically modulate the signaling of other families of morphogens and growth factors.

Results

Characterization of HS-binding probes

FR2c and FR3c interactions with either FGF1 or FGF8b were confirmed (Fig. 1 A) using a heparin-agarose bead (HAB) binding assay where soluble chimeric proteins consisting of the extracellular domain of either FR2c or FR3c fused to alkaline phosphatase (FR2cAP and FR3cAP, respectively) were incubated with HABs in either the presence or absence of FGF1 or FGF8b (Ornitz et al., 1992; Allen et al., 2001). Approximately 25% of FR2cAP and FR3cAP bind HABs in the absence of FGF, confirming their affinity for heparin (Kan et al., 1993); this binding is abolished by 500 mM NaCl washes. Incubation of FR2cAP with either FGF1 or FGF8b results in ~75% binding to HABs, even in 500 mM NaCl. Indeed, 2 M NaCl is required to abrogate ternary complex assembly (unpublished data). In addition, binding is specific, as it is blocked by soluble heparin. FR3cAP also binds both FGF1-heparin and FGF8b-heparin with high affinity (i.e., resistant to 500 mM NaCl washes), although FR3cAP binds less well to FGF1-heparin (50%) than to FGF8b-heparin (75%).

Activation of FR2c and FR3c signaling by either FGF1 or FGF8b (Fig. 1 B) was examined using BaF3 cells, an IL-3 dependent cell line normally negative for both FR and HS (Ornitz et al., 1996). Cells expressing FR2c die in the absence of IL-3, or when supplemented with heparin or FGF1 alone, but survive when treated with FGF1 and heparin. FR2c cells respond equally well to FGF8b and heparin. FR3c expressing cells behave similarly; they proliferate equally when treated with heparin and either FGF1 or FGF8b.

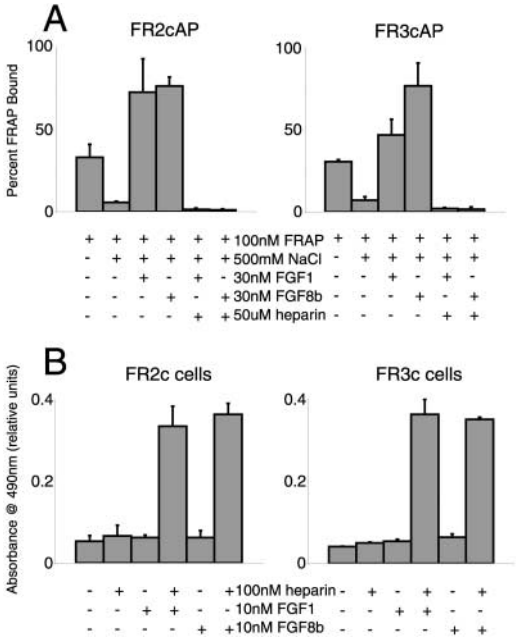

Figure 1. **Binding and activation of FR2c and FR3c by FGF1-heparin and FGF8b-heparin.** (A) Percent FRAP binding to HABs under the conditions shown. (B) Numbers of BaF3 cells expressing either FR2c or FR3c after 48 h in culture medium with the indicated treatments.

Use of HS-binding probes to detect specific HS in situ

To question whether HS mediates these same interactions in situ, LACE was performed on serial sections from mouse embryos at different developmental stages (Allen et al., 2001). Tissue sections from E18 mouse embryos were incubated with either 100 nM FR2cAP or FR3cAP alone or with 30 nM exogenous FGF1 or FGF8b to assess binding to endogenous HS. Varying the concentration of FGF from 3 to 100 nM or FR from 10 nM to 1 μM resulted in identical staining patterns, although of different intensities. Use of lower concentrations resulted in a significant loss of signal, whereas incubation with >100 nM FGF or 1 μM FR1cAP led to nonspecific (i.e., HS independent) binding to sections. Bound FR–alkaline phosphatase (FRAP) was detected using antibodies against its AP portion. The initial focus was in the developing heart and lung because previous work has shown that HS from these two tissues differentially mediates FGF4 and FR interactions (Allen et al., 2001).

HS is identified throughout the embryo (Fig. 2 A) using mAb 3G10, which recognizes the unsaturated uronic acid residue of HS remaining on the core protein after heparinase III digestion. In contrast to their affinity for heparin, neither FR2cAP nor FR3cAP bind to HS in this region (Fig. 2, B and F) or elsewhere in the embryo (unpublished data). This is surprising, because FRs do have an affinity for HS by themselves (Powell et al., 2002). Importantly, FRAP incubation at concentrations as high as 1 μM still results in a failure to bind to HS (unpublished data). However, FRAP incubation together with FGF does result in binding to HS. FR2cAP recognizes FGF1–HS complexes in the heart/lung (Fig. 2 C), representative of binding seen throughout the E18 mouse embryo, indicating that FR2c and FGF1 recognize and interact with HS at these sites.

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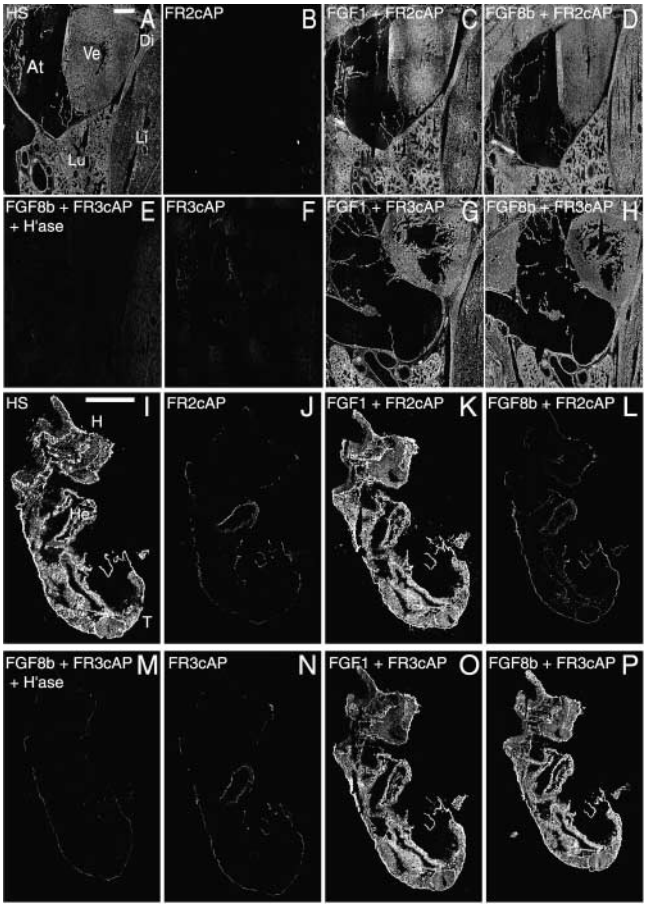

Figure 2. **FGF8b and FR2cAP assemble with E18 HS, but not E8.5 HS.** (A–H) Composite pictures of serial sagittal sections of E18 heart (atrium, At; and ventricle, Ve); lung (Lu), diaphragm (Di), and liver (Li) are shown. (I–P) Sagittal sections of E8.5 mouse embryos are shown as composite images (H, head; He, heart; and T, tail). (A and I) Total HS distribution is identified by mAb 3G10 after treatment with heparin lyases. Sections are incubated with either (B and J) 100 nM FR2cAP or (F and N) 100 nM FR3cAP alone or in the presence of (C, G, K, and O) 30 nM FGF1 or (D, H, L, and P) 30 nM FGF8b. (E and M) FR3cAP is also incubated in the presence of FGF8b on sections pretreated with heparin lyases. FRAP is visualized with antibody to AP. Each treatment was repeated at least three times using different embryos for both stages of development. Bars, 500 μm.

FR2cAP and FGF8b also interact with HS throughout the E18 embryo (Fig. 2 D). As with FR2cAP, FR3cAP also recognizes FGF1-HS (Fig. 2 G) and FGF8b-HS (Fig. 2 H) in the heart and lung, as well as throughout the E18 mouse embryo. As a control for specificity, all binding is abolished if the section is pretreated with a combination of HS lyases (Fig. 2 E); lyase removal of HS blocks binding of any combination of FGF and FRAP described in this paper (unpublished data). In total, these results suggest that HS is capable of mediating complex formation between either FR2c or FR3c and FGF1 or FGF8b.

Although there is no apparent difference in the binding of either receptor to FGF1 or FGF8b in E18 embryos, differences in HS at earlier developmental stages might affect FGF and FR complex assembly. To test this, LACE was performed on sections from E8.5 mouse embryos. Indeed, although FR2cAP recognizes FGF1-HS throughout the embryo at the E8.5 stage (Fig. 2 K), this FR fails to interact with FGF8b-HS at any sites (Fig. 2 L). In contrast, FR3cAP binds throughout the embryo in the presence of either FGF1 (Fig. 2 O) or FGF8b (Fig. 2 P). Again, both FRs fail to bind in the absence of FGF (Fig. 2, J and N) and binding is abolished by HS lyases (Fig. 2 M). Thus, HS from E8.5 embryos mediates the formation of a high affinity complex of FR3cAP with either FGF1 or FGF8b, and FR2cAP with FGF1, but fails to mediate association of FR2cAP and FGF8b.

To determine whether HS from other stage embryos might have yet a different activity, LACE was performed on E9.5 embryos. Here, HS mediates the interactions of FR2cAP with FGF1 (Fig. 3 C), and in contrast to E8.5 embryos, HS also mediates FR2cAP interactions with FGF8b (Fig. 3 D). Perhaps most striking is the global change; HS fails to mediate FGF8b–FR2cAP interactions in E8.5 embryos, but does mediate FGF8b–FR2cAP interactions throughout E9.5 embryos. Global changes in HS also affect FR3cAP interactions in these embryos. FR3cAP fails to recognize FGF1-HS except weakly in certain basement membranes near the surface ectoderm (Fig. 3 G), opposite to that seen in E8.5 embryos, where FR3cAP binds FGF1-HS at all sites. However, FR3cAP binds FGF8b-HS throughout E9.5 embryos (Fig. 3 H).

LACE was also used 2 d later in E12 embryos. FR2cAP binds FGF1-HS throughout the embryo (Fig. 3 K). In contrast, it fails to bind FGF8b-HS in the majority of the embryo (Fig. 3 L), similar to its binding profile at E8.5. FR2cAP does bind FGF8b–HS complexes weakly in select basement membranes surrounding the brain and spinal cord as well as in the surface ectoderm. A potential explanation for this failure is a lack of FGF8b binding to HS. However, FR3cAP does bind FGF8b-HS throughout the E12 embryos (Fig. 3 P). Thus, FGF8b interacts with HS in E12 embryos, at least when in a ternary complex with FR3cAP. Examination of FR3cAP binding to FGF1-HS in these embryos reveals a more tissue-specific HS binding pattern (Fig. 3 O). The FR binds FGF1-HS in multiple sites, including the liver, neuroepithelium, and the branchial arch. However, FR3cAP fails to bind FGF1-HS in either the atrium or the ventricle of the heart. FR3cAP also binds FGF1-HS less strongly in the somites, although there is some experimental variation in this staining.

Each FGF-FR pair seeks a distinct HS domain

In total, these results suggest that developmental stage–specific changes in HS regulate FR2cAP and FR3cAP complex assembly with either FGF1 or FGF8b, apparently due to changes in the structure of HS. Furthermore, these data suggest that each FR and FGF pair seeks a different HS domain, even though this was not initially observed at the E18 stage. To test whether the FGF-FR pairs are indeed binding to different HS domains in E18 embryos, selectively desulfated heparins were used to compete binding to endogenous HS at this stage. The results focus on the lung, although similar binding is seen throughout the embryo. Sections were preincubated with FGF, creating preformed FGF–HS complexes on the section, followed by FRAP alone, or in the presence of heparin, 2-*O*-desulfated (2-ODS) heparin, or 6-ODS heparin (Fig. 4). When FR2cAP is incubated with sections

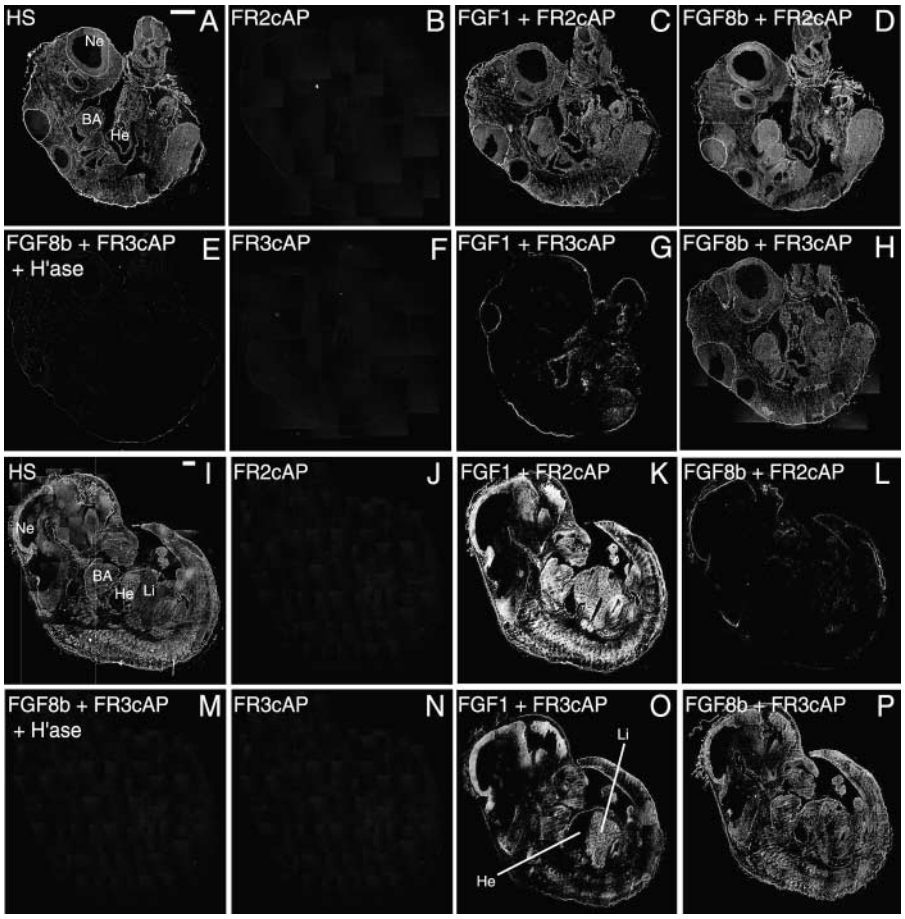

Figure 3. **Global changes in HS-mediated assembly of FGF1-FR3cAP and FGF8b–FR2cAP complexes in E9.5 and E12 mouse embryos.** Composite images of sagittal sections of (A–H) E9.5 and (I–P) E12 embryos are shown. Detection of (A and I) total HS with mAb 3G10; (B and J) binding of 100 nM FR2cAP alone; (C and K) binding of FR2cAP in the presence of 30 nM FGF1 or (D and L) 30 nM FGF8b; (F and N) 100 nM FR3cAP alone; (G and O) binding of FR3cAP in the presence of 30 nM FGF1 or (H and P) 30 nM FGF8b. (E and M) Sections pretreated with heparin lyases and incubated with 100 nM FR3cAP and 30 nM FGF8b. He, heart; Li, liver; BA, branchial arch; and Ne, neuroepithelium. Experiments were repeated at least three times with different embryos to confirm the results. Bars, 500 μm.

treated previously with FGF8b, the FR binds avidly to the FGF8b–HS complexes in the lung (Fig. 4 A). However, incubation of FR2cAP together with heparin blocks the binding, suggesting that the heparin may compete by occupying the heparin-binding domain (HBD) on the FR (Fig. 4 B). Incubation of FR2cAP with either 2-ODS heparin (Fig. 4 C) or 6-ODS heparin (Fig. 4 D), results in no competition. This tentatively suggested that FR2cAP does not interact with heparin that lacks either 2-*O*-sulfation or 6-*O*-sulfation. Turning to FR3cAP, this FR also binds strongly to FGF8b-HS in the lung (Fig. 4 E). Again, incubation of FR3cAP with heparin results in competition of binding of FR3cAP to the section (Fig. 4 F). Interestingly, incubation of FR3cAP with 2-ODS heparin also competes binding (Fig. 4 G), whereas it does not compete FR2cAP. However, like FR2cAP, FR3cAP binding to FGF8b-HS is not competed by 6-ODS heparin (Fig. 4 H). These results tentatively suggested that FR3cAP does not require 2-*O*-sulfation in order to interact with heparin, but does require 6-*O*-sulfation. Overall these results indicate that these FR and FGF pairs do require different sulfation patterns for their interaction, and tentatively suggest specificities of the FR HBDs.

To confirm that this competition identifies differences in the FR HBDs, heparin competition of FRAP binding to FGF1-HS was examined. Here, the competition with FR binding to FGF1 would be expected to be identical to FGF8b if only the FR is being competed by the modified heparin. As expected, FR2cAP and FR3cAP both bind

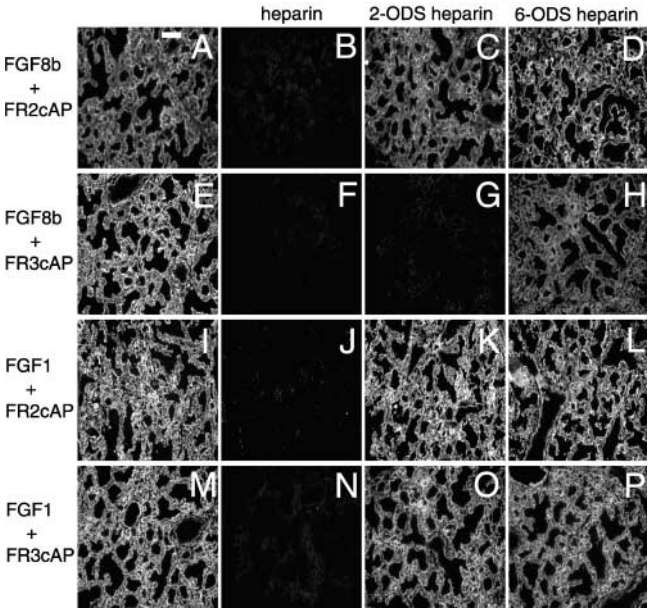

Figure 4. **Heparin competition of FGF–FRAP complexes from HS in E18 mouse embryos.** Detection of FR2cAP (A–D and I–L) or FR3cAP (E–H and M–P) binding to E18 lung preincubated with (I–P) 30 nM FGF1 or (A–H) 30 nM FGF8b. Incubation of (A, E, I, and M) 100 nM FRAP alone, or with (B, F, J, and N) 50 nM heparin, (C, G, K, and O) 50 nM 2-ODS heparin, or (D, H, L, and P) 50 nM 6-ODS heparin. Bar, 100 μm.

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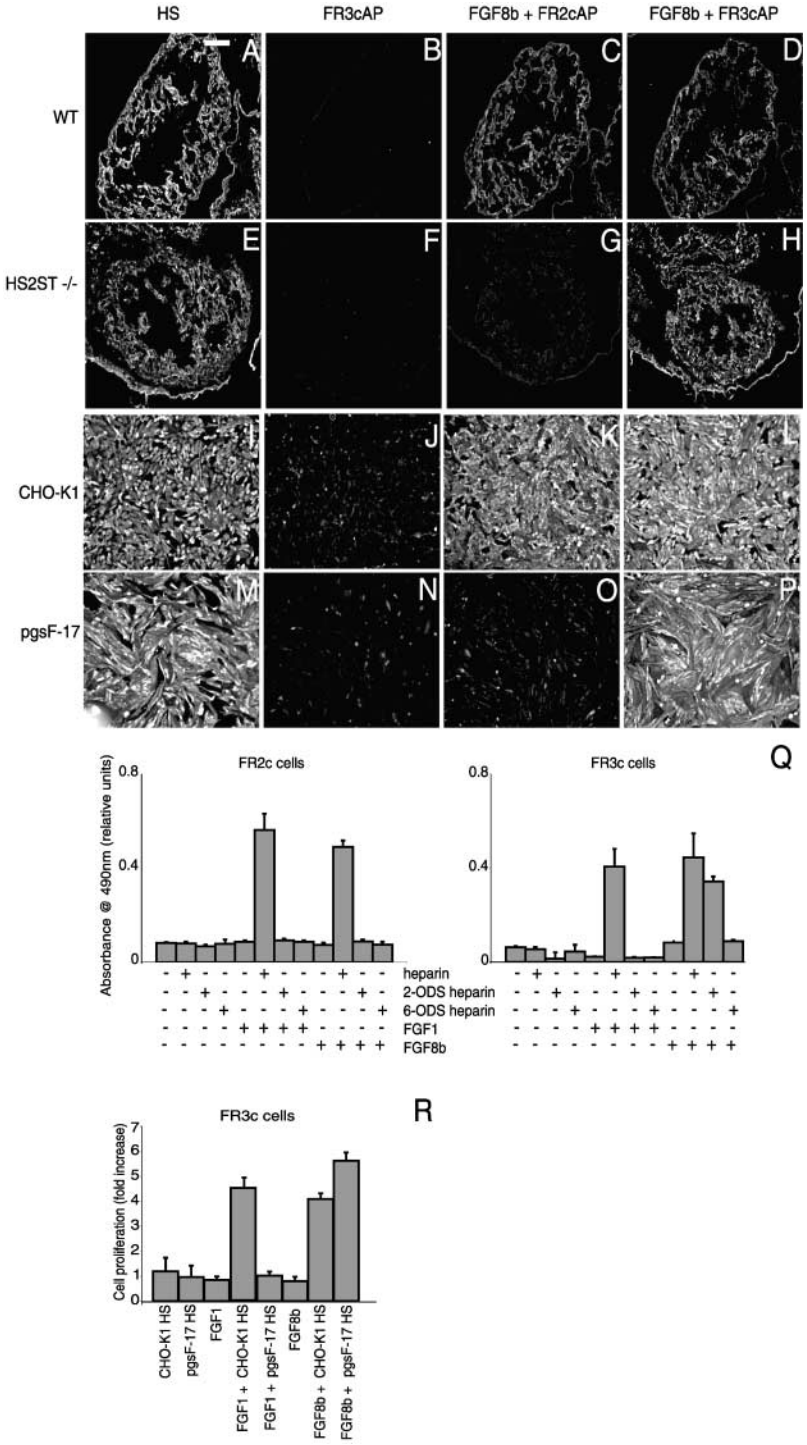

Figure 5. **FGF8b and FR3c form an active signaling complex in the absence of 2-*O*-sulfation.** Assembly of FGF–HS–FRAP complexes on (A–D) E10 *Hs2st*$^{+/+}$ and (E–H) *Hs2st*$^{-/-}$ embryos and wild-type (I–L) CHO-K1 and (M–P) pgsF-17 cells. mAb 3G10 identification of total HS (A, E, I, and M). Binding of (B, F, J, and N) 100 nM FR3cAP after incubation alone, or with (D, H, L, and P) 30 nM FGF8b. Binding of 100 nM FR2cAP after incubation with (C, G, K, and O) 30 nM FGF8b. Bar, 100 μm. (Q) Quantification of BaF3 cells expressing either FR2c or FR3c after 48 h in the treatments shown. (R) Proliferative response of BaF3 cells expressing FR3c to FGF1 or FGF8b and HS isolated from either CHO-K1 or pgsF-17 cells. (Q and R) Error bars represent the SD for quadruplicate samples in a single experiment, and are representative of three independent experiments.

FGF1-HS (Fig. 4, I and M), and binding of both FRAPs is competed with heparin (Fig. 4, J and N). Surprisingly, neither FR2cAP nor FR3cAP binding is competed with either 2-ODS heparin (Fig. 4, K and O) or 6-ODS heparin (Fig. 4, L and P), suggesting that 2-*O*-sulfates and 6-*O*-sulfates are necessary for both FR2c and FR3c assembly with FGF1. This contrasts with FGF8b, where 2-*O*-sulfates appear unnecessary for binding to FR3c. This unexpected finding suggests that the HS binding requirements of specific FRs alone are not being tested. One explanation is that the heparin competes FGF binding to sections. However, for this to be true, 2-ODS heparin should compete both FR2cAP and FR3cAP binding to FGF8b-HS, which it does not. Another possibility is that these heparins selectively compete by inserting into a binding pocket generated only when the FGF and FR are assembling into a complex. This possibility is consistent with the data, and was examined further.

To test FR3cAP and FGF8b interactions in the absence of 2-*O*-sulfates, complex assembly with HS from mouse embryos that lack 2-*O*-sulfation was examined using LACE. Mice expressing a gene trap mutation in *Hs2st* do not ex-

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press HS 2-*O*-sulfotransferase (HS2ST) and synthesize HS lacking 2-*O*-sulfation (Bullock et al., 1998). FR2cAP and FR3cAP binding to FGF8b-HS in tissue sections from both *Hs2st*$^{+/+}$ and *Hs2st*$^{-/-}$ E10 embryos from the same litter was examined. This focused on the primitive ventricle of the heart. Although FR2cAP interacts with FGF8b-HS in the *Hs2st*$^{+/+}$ embryos (Fig. 5 C), it fails to bind FGF8b-HS in the *Hs2st*$^{-/-}$ embryos (Fig. 5 G). In contrast, FR3cAP interacts with FGF8b-HS in both the *Hs2st*$^{+/+}$ (Fig. 5 D) and *Hs2st*$^{-/-}$ (Fig. 5 H) embryos. Thus, FR3cAP and FGF8b form a ternary complex with HS that lacks 2-*O*-sulfation, but FR2cAP and FGF8b cannot.

These results were extended using CHO cells that also fail to express HS2ST (pgsF-17 cells; Bai and Esko, 1996). As in the *Hs2st* embryos, FR2cAP bound to FGF8b-HS on wild-type CHO-K1 cells (Fig. 5 K), but failed to bind FGF8b-HS on 2-*O*-sulfate deficient pgsF-17 cells (Fig. 5 O). FR3cAP binding on these cells was also equivalent to that seen in the *Hs2st* embryos, interacting with FGF8b-HS on both CHO-K1 cells (Fig. 5 L) and pgsF-17 cells (Fig. 5 P).

To confirm that LACE accurately depicts interactions necessary to form an active signaling complex, the proliferative response of BaF3 cells expressing either FR2c or FR3c was examined (Fig. 5 Q). As expected from LACE, FR2c cells do not respond to FGF-8b in the presence of either 2-ODS heparin or 6-ODS heparin. Thus, FGF8b-FR2c requires heparin or HS containing both 2-*O*-sulfation and 6-*O*-sulfation to form an active signaling complex. FR3c cells also fail to respond to FGF8b and 6-ODS heparin. However, they do respond to FGF8b and 2-ODS heparin. This confirms that although FGF8b-FR3c requires heparin bearing 6-*O*-sulfates to assemble and signal, 2-*O*-sulfation is not necessary. The response of FR2c and FR3c cells to FGF1 was examined next (Fig. 5 Q). Neither cell line responds to FGF1 in combination with either 2-ODS heparin or 6-ODS heparin.

To directly correlate the formation of FGF–HS–FR complexes on cells and sections with the formation of an active signaling complex, HS was also isolated from both CHO-K1 and pgsF-17 cells for use in proliferation assays (Fig. 5 R). FR3c cells respond to either FGF1 or FGF8b with HS from CHO-K1 cells. In contrast, FR3c cells respond to FGF8b with HS from pgsF-17 cells, but fail to respond to FGF1 with this HS. Importantly, these results correspond exactly to the formation of ternary complexes of FGF–HS–FR on fixed monolayers of these cells.

FGF-FR pairs interact synergistically to identify an HS domain

There are three potential explanations for the failure of FGF1 to assemble with either FR2c or FR3c and HS in the absence of 2-*O*-sulfation or 6-*O*-sulfation. The first is that FGF1 requires 2-*O*-sulfates and 6-*O*-sulfates in order to bind HS; this is consistent with previously published results (Guimond et al., 1993; Kreuger et al., 2001). A second explanation is that both FRs require 2-*O*-sulfates and 6-*O*-sulfates in order to bind HS; however, this is inconsistent with the results using FGF8b described previously (Figs. 4 and 5). A third explanation is that formation of the ternary complex uses sulfation requirements not exhibited by either the FGF or the FR binding individually.

To test whether the 2-*O*-sulfation requirement for FRAP binding to FGF1 reflects a requirement of the FGF alone, the FR alone, or a requirement unique to this ternary complex, the binding of FR2cAP, FR2bAP, and FR3cAP to FGF1 was examined (Fig. 6). Here, FR2b and FR2c were compared because these two splice variants of FR2 both recognize FGF1 and have identical HBDs because splicing does not alter the amino acid sequence in this region of DII. A recent paper also indicates that FGF1 signals through FR2b in the absence of either 2-*O*-sulfation or 6-*O*-sulfation (Ostrovsky et al., 2002). Binding was examined using the *Hs2st* embryos as well as the pgsF-17 cells. In *Hs2st*$^{+/+}$ and *Hs2st*$^{-/-}$ embryos, FR2bAP fails to bind in the absence of FGF1 (Fig. 6, A and E), but does assemble with FGF1 and HS expressed by either the *Hs2st*$^{+/+}$ or the *Hs2st*$^{-/-}$ embryos (Fig. 6, B and F); thus, both FR2b and FGF1 in this complex interact with HS that lacks 2-*O*-sulfates. In contrast, FR2cAP and FR3cAP both fail to bind FGF1-HS in the *Hs2st*$^{-/-}$ embryos (Fig. 6, G and H), although they do bind FGF1-HS in the *Hs2st*$^{+/+}$ embryos (Fig. 6, C and D). This result is confirmed using CHO-K1 cells or 2-*O*-sulfate deficient pgsF-17 CHO cells (Fig. 6, I–R). In addition, FGF1 binding to HS in either cell type was directly examined using an FGF1-specific antibody. Although FGF1 binds HS in CHO-K1 cells (Fig. 6 I), it fails to bind in the pgsF-17 cells (Fig. 6 N). FGF1 does bind pgsF-17 HS, however, when incubated simultaneously with FR2bAP (Fig. 6 P, inset).

To confirm that FR2bAP and FGF1 interact in the absence of 2-*O*-sulfation, the ability of 2-ODS heparin to compete FGF1 and FR2bAP binding to HS was examined (Fig. 6, S–V). FR2bAP and FGF1 assemble with lung HS in E18 embryos (Fig. 6 S), but binding is abrogated when incubated with heparin (Fig. 6 T). In addition, binding is also abrogated when FGF1 and FR2bAP are incubated with either 2-ODS heparin (Fig. 6 U) or 6-ODS heparin (Fig. 6 V). To correlate binding with activity, the response of BaF3 cells expressing FR2b was examined in response to treatment with FGF1 and heparin, 2-ODS heparin, or 6-ODS heparin (Fig. 6 W). These data show that FR2b cells respond to FGF1 with any of the heparins used. Overall, these data support the third explanation advanced earlier, namely, that ternary complex formation invokes binding requirements distinct from the FGF or the FRAP HBDs alone. Therefore, although an FGF and FR may separately have identifiable HS binding requirements, the HS requirements necessary to simultaneously form a ternary complex may be different.

FR2bAP and FR2cAP interactions with FGF1 were further examined with HS that lacks 2-*O*-sulfation by preincubating the FGF alone with the HS, followed by washing before addition of the FRAP (Fig. 7). HS clearly retains FGF1 and this FGF–HS complex assembles with either FR2cAP or FR2bAP in the *Hs2st*$^{+/+}$ embryos (Fig. 7, A and B). However, no assembly is detected in the *Hs2st*$^{-/-}$ embryos (Fig. 7, D and E). Identical results were observed in wild-type (Fig. 7, G and H) and pgsF-17 CHO cells (Fig. 7, J and K). This suggests that FGF1 does require 2-*O*-sulfation in order to bind HS with appreciable affinity by itself. However, this requirement does not exist if FGF1 assembles with certain

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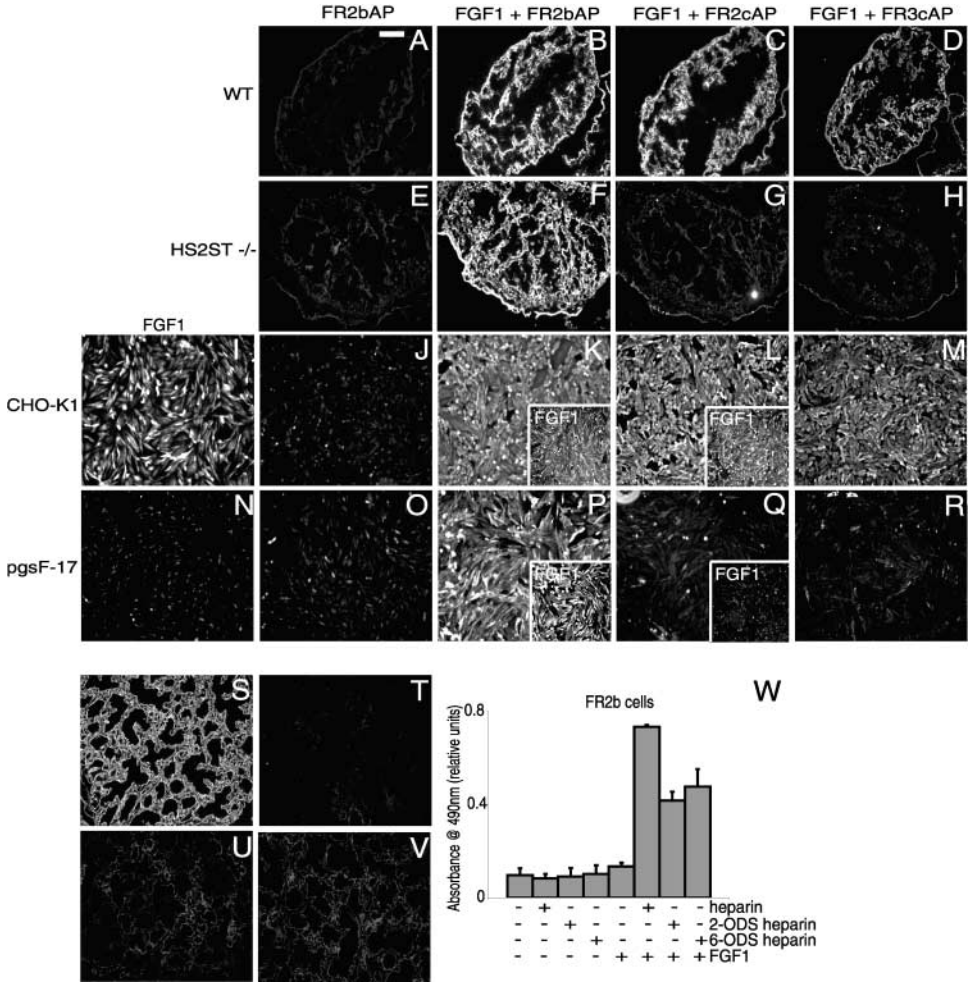

Figure 6. **FGF1–FR2bAP complex assembly in the absence of 2-*O*-sulfation.** Assembly of FGF–HS–FRAP complexes on (A–D) E10 $Hs2st^{+/+}$ and (E–H) $Hs2st^{-/-}$ embryos and wild-type (I–M) CHO-K1 and (N–R) pgsF-17 cells. Detection of FRAP after binding of (A, E, J, and O) 100 nM FR2bAP alone or (B, F, K, and P) 100 nM FR2bAP, (C, G, L, and Q) 100 nM FR2cAP, or (D, H, M, and R) 100 nM FR3cAP in the presence of 30 nM FGF1. FGF1 binding alone (I and N) or in the presence of FR2b (K and P, insets) or FR2c (L and Q, insets) is detected. Formation of FGF1 and FR2bAP complexes is detected in (S) E18 mouse lung alone or in the presence of (T) 50 nM heparin, (U) 50 nM 2-ODS heparin, or (V) 50 nM 6-ODS heparin. (W) The proliferative response of BaF3 cells expressing FR2b to FGF1 and heparin, 2-ODS heparin, or 6-ODS heparin is measured. Bar, 100 μm. Error bars represent the SD for quadruplicate samples in a single experiment, and are representative of three independent experiments.

FRs, such as FR2b where a ternary complex forms in the absence of 2-*O*-sulfation. Interestingly, FR3cAP interacts with preincubated FGF8b-HS in both the $Hs2st^{+/+}$ (Fig. 7 C) and $Hs2st^{-/-}$ embryos (Fig. 7 F), as well as in the wild-type (Fig. 7 I) and pgsF-17 CHO cells (Fig. 7 L). Thus, FGF8b does not require 2-*O*-sulfation to bind HS independent of an FR.

Discussion

Identification of developmental stage–specific HS

The focus of this paper was to examine whether HS regulates ligand–receptor interactions in a developmental stage–specific manner. LACE was used with four different sets of FGF-FR probes to survey the developing mouse embryo at various gestational stages. Although the FGF-FR pairs interact with heparin in vitro, and with HS in vivo in E18 stage embryos, dramatic differences are seen at several earlier stages of development (E8.5, E9.5, and E12). Importantly, each FGF-FR pair examined has unique HS-binding requirements, which suggest that within HS there are distinct domains that act as highly specific regulators of morphogen signaling.

These results provide an important insight into HS regulation of FGF signaling and potentially that of other HS-dependent morphogens. Specifically, LACE now provides an ability to assess HS in developmental processes where expression of FGFs and FRs are known, but the activity of the HS, a critical regulatory partner in these signaling interactions, is unknown. One such process is heart development, where both FGF1 and FGF8b play important roles. FGF1 (as well as FGF2 and FGF4) impacts the differentiation of chick precardiac mesenchyme and also appears later (stages 18–24) during chick heart chamber development (Zhu et al., 1996), which corresponds with E9–11 in the mouse. FGF8 affects the induction of cardiogenic precursors during both zebrafish and chick heart development (Reifers et al., 2000; Alsan and Schultheiss, 2002). FGF8 also regulates the establishment of left–right asymmetry in the developing mouse heart (Meyers and Martin, 1999). In addition, there are reports that the in-

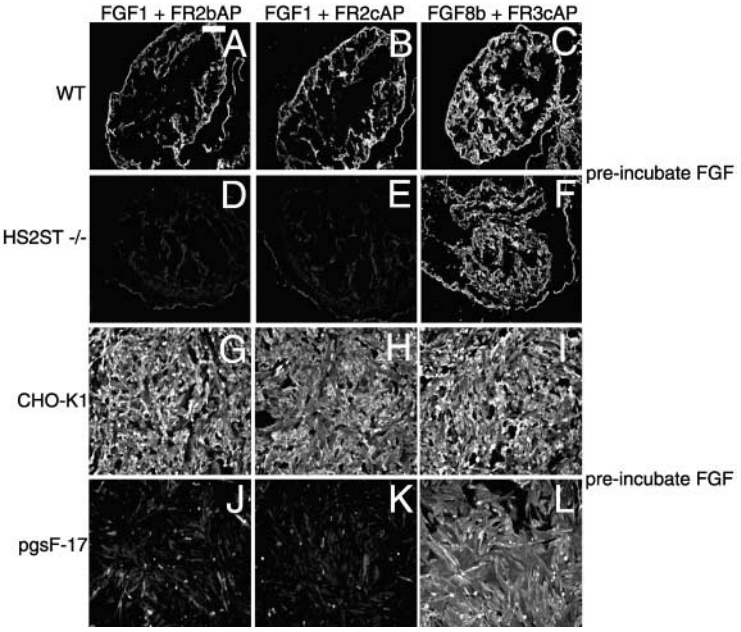

Figure 7. **FGF1 fails to bind HS in the absence of 2-*O*-sulfation.** Sections are from (A–C) E10 *Hs2st*$^{+/+}$ or (D–F) *Hs2st*$^{-/-}$ embryos or fixed (G–I) CHO-K1 or (J–L) pgsF-17 cells. Binding of (A, D, G, and J) FR2bAP or (B, E, H, and K) FR2cAP to sections or cells preincubated with 30 nM FGF1 is shown. Bound FR3cAP is detected after incubation of 100 nM FR3cAP on sections or cells preincubated with (C, F, I, and L) 30 nM FGF8b. Bar, 100 μm.

hibition of proteoglycan synthesis disrupts heart development (Walsh and Stainier, 2001) and left–right asymmetry in the heart (Yost, 1992). This corresponds with our current and prior work (Allen et al., 2001), which suggests that HS in the developing heart displays a high degree of specificity in mediating FGF and FR interactions. In particular, the inability of FGF1 and FR3c to interact in E12 mouse heart compared with restored binding of this FGF-FR pair in E9.5 mouse heart suggests that HS may specifically regulate FGF1 signaling in the heart at different developmental stages.

A second process that requires FGF signaling is limb development. Specifically, FGF8b is expressed in the apical ectodermal ridge at E9-E12.5 (Crossley and Martin, 1995) and targeted inactivation of *FGF-8* in the mouse clearly demonstrates that FGF8 is essential for normal limb development (Sun et al., 2002). However, an important issue that remains is the identification of the FR through which FGF8 signals in the limb. Inactivation of the *Fgfr2* gene results in the loss of limb formation in the mouse (Arman et al., 1999), although recent evidence demonstrates that the limb phenotype resulting from inactivation of *Fgfr2* targets FR2b and not FR2c (Revest et al., 2001; Eswarakumar et al., 2002). Genetic studies suggest that FR1c is important for limb development (Deng et al., 1997), although it is not likely that FGF8 can signal through this receptor because activity studies have shown that micromolar concentrations of FGF8b are needed to transduce a signal through FR1c (MacArthur et al., 1995). Further studies will be necessary to identify the FR through which FGF-8b signals during limb development, but the data in this paper suggest that, if FGF8b and FR2c do interact in the limb, there is a specific developmental window during which the HS is appropriate, namely, at E9.5 stage of development.

FGF signaling also regulates brain development (Dono, 2003). Both FGF1 and FGF8, as well as FR2c and FR3c are expressed in a tissue-specific and developmental stage–specific manner during brain development (Peters et al., 1993; Reid and Ferretti, 2003). Although the role of FGF1 expression in the brain remains unclear, data suggest that FGF8 is critical for correct patterning of the neocortex (Fukuchi-Shimogori and Grove, 2001), as well as cell survival in the developing forebrain and midbrain (Chi et al., 2003). In addition, FR3 may be important for postnatal brain development, particularly for the terminal differentiation of oligodendrocytes (Oh et al., 2003). Our data suggest that FGF8 and FR3c are able to signal throughout the brain during development, but that FGF8 signaling through FR2c, as well as FGF1 signaling through both FR2c and FR3c, is restricted by the expression of developmental stage–specific HS in the brain.

However, perhaps the most striking finding is not just the existence of these developmental changes but also the global manner in which they occur. That is, HS changes throughout the entire embryo. This is not necessarily surprising in a physical sense, because the embryo is undergoing tremendous growth and remodeling at these stages. However, it is surprising that the synthesis of specific HS domains is tightly and uniformly regulated throughout all embryonic tissues. Importantly, the expression of HS biosynthetic enzymes does change during development (Aikawa et al., 2001; Ford-Perriss et al., 2002), as does the expression of core proteins to which HS is attached (Kim et al., 1994; Litwack et al., 1998). However, these findings focus on individual tissues and provide no indication that such changes are global. Extracellular sulfatases, which act to modify HS already present at the cell surface or in the ECM are also reported (Dhoot et al., 2001; Morimoto-Tomita et al., 2002); however, these also appear localized in their expression. Thus, although changes in the expression of enzymes or core proteins may suffice to explain tissue-specific changes in HS structure, none of them appears adequate to explain the global changes in HS structure shown here. Whatever the explanation, it appears to be a highly coordinated regulation that encompasses all of the tissues in the embryo.

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HS requirements for FGF–FR complex assembly and signaling

Because HS-mediated FGF–FR complex assembly varies with each developmental stage, a major objective of this paper was to identify the HS requirements that determine these interactions. Strikingly, individual FGF-FR pairs exhibit unique HS binding requirements for ternary complex assembly. In fact, no two FGF-FR pairs display the same combination of (a) HS-binding patterns in developing embryos and (b) sulfation requirements necessary for ternary complex assembly and signaling (Table I). Importantly, the binding requirement of the FR changes depending on the identity of the FGF, even between FR2b or FR2c; two splice variants of the same FR gene product in which the HBD is presumably identical. This suggests that the HS-binding pocket displayed by an FGF-FR pair is unique to that pair and may or may not reflect the binding properties of either protein alone. Perhaps the interaction of the FR and FGF exposes or masks amino acids that contribute to HS binding in either protein. This may be a physical masking produced by the protein–protein interaction, or it might involve slight conformational changes in the proteins. In fact, a recent crystallographic study demonstrates that FR2b does indeed undergo a conformational change upon binding to FGF10 (Yeh et al., 2003).

Role of 2-*O*-sulfation in FGF signaling

The interactions of FGF8b–FR3c and FGF1–FR2b with HS lacking 2-*O*-sulfation provide insight into other works. First, the phenotypic effects of the *Hs2st* gene knockout in mice (Bullock et al., 1998), are relatively mild given the important role of FGF signaling during development, and the reliance of FGF signaling on HS. However, it is now clear that 2-*O*-sulfation is dispensable for at least two FGF–FR interactions, and possibly more. Of course, these mice also show an increase in both *N*- and 6-*O*-sulfation, suggesting a potential mechanism that compensates for the loss of 2-*O*-sulfation (Merry et al., 2001). Nonetheless, our data show that FGF1 and FR2c are unlikely to interact in these mice.

A second insight is provided by the assembly of FGF1 and FR2b with HS in the absence of 2-*O*-sulfation. Conflicting reports exist in the literature, showing that FGF1 requires 2-*O*-sulfation to bind HS and signal through an FR (Guimond et al., 1993; Kreuger et al., 2001), or showing that FGF1 can signal through FR2b in the absence of 2-*O*-sulfation (Ostrovsky et al., 2002). Our data provide an explanation for the apparent discrepancy, namely, that FGF1 by itself does require 2-*O*-sulfation to interact with HS, as well as to interact in a complex with HS and FR2c, but does not require 2-*O*-sulfation to interact in a complex with HS and FR2b. In other words, the binding requirements of FGF1 alone or for its signaling through one FR do not suffice to predict its signaling requirements with another FR. Importantly, a recent paper demonstrates that FGF1 and FR1c display similar HS binding requirements (Wu et al., 2003).

Proposed model of FGF–HS–FR complex assembly

Based on the insights that this paper provides into the physical assembly of an FGF–HS–FR complex, we propose two related models for the formation of a high affinity FGF–HS–FR complex. In the first model, specific HS domains exist that contain the appropriate sulfation pattern necessary for FR recognition. This is based on the data presented in this paper, as well as the work of other labs, which have shown that FRs appear to specifically recognize different HS domains (Guimond and Turnbull, 1999; Powell et al., 2002). There are also specific HS domains that are selectively recognized by FGFs. However, ternary complex formation occurs only when an HS domain contains the sulfation pattern necessary for both FGF and FR recognition. In this model (additive), the HS domain is simply a sum of the individual FGF and FR binding requirements. In the second model (synergistic), there are again FGF and FR-specific HS domains, which are selectively recognized by one component independently of the other. However, the HS domain that mediates ternary complex formation contains a different set of sulfation requirements that is dictated by the binding pocket formed by specific FGF-FR pairs. It is this synergistic model that fits the data presented for the FGF1–FR2b interactions. However, for FGF8b, either model could be true, given the data available. Therefore, more experiments will be required to determine if the synergistic model presented here is a general feature of all FGF–FR interactions, or if it is specific to FGF1.

HS regulation of growth factor signaling

Published reports from this lab and others have demonstrated the usefulness of LACE to elucidate tissue-specific

Table I. **FGF-FR pairs display unique combinations of developmental HS-binding patterns and sulfation requirements**

	Developmental stage				Sulfation requirement[a]	
FGF-FR pair	**E8.5**	**E9.5**	**E12**	**E18**	**2-*O*-sulfation**	**6-*O*-sulfation**
FGF1-FR2b	ND	ND	ND	ND	−	−
FGF1-FR2c	+	+	+	+	+	+
FGF1-FR3c	+	−	±[b]	+	+	+
FGF8b-FR2c	−	+	−	+	+	+
FGF8b-FR3c	+	+	+	+	−	+

ND, no data.
[a]Sulfation requirements are based on FGF–FR complex assembly and signaling with heparin or HS that lacks either 2-*O*-sulfation or 6-*O*-sulfation. No data are available regarding FGF-FR signaling in the absence of both 2-*O*-sulfation and 6-*O*-sulfation. Thus, although FGF1-FR2b interact in the absence of either 2-*O*-sulfation or 6-*O*-sulfation, it is unknown if complex assembly occurs with the simultaneous loss of both 2-*O*-sulfation and 6-*O*-sulfation.
[b]FGF1-FR3c interact at multiple sites in E12 mouse embryos, but fail to interact in the heart, and interact only weakly in somites.

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interactions between FGFs, FRs, and HS (Chang et al., 2000; Allen et al., 2001), as well as between other ligands and carbohydrates (Desnoyers et al., 2001; Rubin et al., 2002). This paper now also demonstrates the utility of LACE to identify developmental stage–specific HS domains that regulate ligand–receptor interactions. The identification of these domains also suggests that, given the multitude of morphogen interactions mediated by HS, developmental stage–specific changes in HS may also affect the functions of these proteins. Studies in *Drosophila melanogaster* have demonstrated that HS regulates the function of a number of developmental signaling molecules, including Hh, Wg, and Dpp (Nybakken and Perrimon, 2002). Importantly, HS may not only regulate ligand–receptor interactions, as described here for FGF signaling, but may also regulate morphogen gradient formation during development. Data from other laboratories have shown that alterations in HS have dramatic effects on the accumulation of Wg during development (Giraldez et al., 2002). Additional reports demonstrates that Hh interactions with HS regulate its movement during development (The et al., 1999) and that these interactions change during early postnatal mouse development (Rubin et al., 2002), suggesting that indeed other morphogens may be subject to the same degree of regulation as FGF family members. A critical question that remains is what affect the developmental stage–specific changes in HS identified in this paper have on the function of these signaling molecules. It may be that these molecules are less sensitive to changes in HS structure than FGFs such that subtle changes in HS may have no effect on their activity. A more intriguing possibility is that some or all of these proteins are sensitive to such changes in HS, so that developmental alterations in HS structure play a crucial role in modulating the numerous cellular processes regulated by these morphogens.

Materials and methods

Image acquisition and manipulation

All images were acquired at RT using Image-Pro® Plus version 1.3 from a microscope (model Microphot-FX; Nikon) with an attached Photometrics Image Point™ scientific cooled CCD video camera. mAb 3G10 binding was detected using Cy3-conjugated donkey anti–mouse secondary antibodies (Molecular Probes). Bound FRAP was recognized with Alexa 546–conjugated donkey anti–rabbit secondary antibodies (Molecular Probes). FGF1 was identified using Alexa 488–conjugated donkey anti–goat secondary antibodies. All slides were mounted with a coverslip in the aqueous, nonfluorescing mounting medium, Immu-mount (Thermo Shandon). Pictures for composite images (Figs. 2 and 3) were obtained using a 6.3× Neofluar objective (Carl Zeiss MicroImaging, Inc.) with an NA of 0.2. All other pictures (Figs. 4–7) were acquired with a 10× Fluor objective (Nikon) with an NA of 0.5. Images were processed (brightness, contrast, and color balance adjustments only) in Adobe Photoshop version 7.0.

FRAP fusion proteins

Soluble FRAP fusion proteins consist of the extracellular three Ig-like loop domains of either the IIIb or IIIc splice variants of FR2 or the IIIc splice variant of FR3 fused to the NH_2 terminus of human placental-AP (FR2bAP, FR2cAP, and FR3cAP, respectively; Ornitz et al., 1992). FRAP cDNAs were provided by D. Ornitz (Washington University, St. Louis, MO). Purification of FRAP fusion proteins has been described previously (Allen et al., 2001; Rapraeger, 2002).

FGF ligands

The SG13009 strain of *Escherichia coli* transformed with the pQE16 expression plasmid containing the cDNA encoding murine FGF8b was the gift of C. MacArthur (Washington University). Purification of bacterially expressed His-tagged FGF8b has been described previously (MacArthur et al., 1995; Rapraeger, 2002). Because there have been reports of His-tagged proteins binding nonspecifically to HS (Lacy and Sanderson, 2002), experimental results with His-tagged FGF8b were confirmed using untagged, commercially available recombinant human FGF8b (Peprotech). Recombinant human FGF1 was the gift of B. Olwin (University of Colorado at Boulder, Boulder, CO) or was obtained commercially (Intergen).

HAB binding assay

FRAP binding to FGF–heparin was performed using HABs (Bio-Rad Laboratories). FRAP was incubated in Hepes-buffered RPMI + 10% CS + 4 mM L-glutamine at 100 nM with 10-μl HABs in either the absence or the presence of 30 nM human recombinant FGF1 or 30 nM human recombinant FGF8b for 1 h at RT on a rotator (Scientific Equipment Products). HABs bearing FRAP were washed with either TBS or TBS containing 500 mM NaCl and loaded into 96-well plates with an equal volume of AP substrate solution; AP activity was determined by absorbance at 405 nm. Bound FRAP was calculated as a percentage of the total FRAP added to each treatment group.

LACE assay

LACE assays were performed as described previously (Friedl et al., 2001; Rapraeger, 2002). Embryos were harvested from timed pregnant CD-1 mice (Charles River Laboratories) and frozen in optimal cutting temperature compund (VWR International). *Hs2st*$^{+/+}$ and *Hs2st*$^{-/-}$ embryos were the gift of C. Alexander (University of Wisconsin). Embryos were staged based on the criteria of the Edinubrgh Mouse Atlas Project (http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.html) and the work of Kaufman (1992). For E8.5 embryos, turning of the embryos occurred as the result of physical manipulation during the embedding process. 5-μm fresh frozen sections from E8.5, E9.5, E12, or E18 embryos were obtained and prepared as described previously (Allen et al., 2001). For analysis of FRAP binding to endogenous HS, frozen sections were incubated with 30 nM recombinant human FGF1 or FGF8b and 100 nM FR2cAP or FR3cAP in Hb-RPMI containing 10%CS and 4 mM L-glutamine. Bound FRAP was identified by incubation with polyclonal rabbit anti-PLAP (Biomeda Corp.) for 30 min. FGF1 was detected using a goat anti-FGF1 antibody (R&D Systems).

Heparin competition experiments

To examine heparin or chemically desulfated heparin competition of FRAP binding, tissue sections were preincubated for 1 h with 30 nM FGF, followed by triplicate washes with TBS. 100 nM FRAP and 50 nM porcine intestinal mucosa heparin (Sigma-Aldrich), 50 nM 2-ODS heparin (Neoparin), or 50 nM 6-ODS heparin (Guimond et al., 1993) were incubated together on the sections for 1 h at RT.

Cell culture

BaF_3 lymphoid cells expressing FR2b, FR2c, and FR3c (FR2b7, FR2c2, and FR31c cells, respectively) were provided by D. Ornitz. FR2b7, FR2c2, and FR31c cells were cultured in RPMI 1640 (GIBCO BRL), 10% FCS (Hyclone), 10% WEHI-3–conditioned medium, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.0035% β-mercaptoethanol.

Wild-type CHO-K1 and mutant pgsF-17 cells were the gift of J. Esko (University of California at San Diego, La Jolla, CA). Cells were grown in Ham's F-12 (GIBCO BRL), 10% FBS (Tissue Culture Biologicals), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell binding experiments

CHO-K1 and pgsF-17 cells were plated at a density of 10^5 cells/well on 8-well TC chamber slides (Fisher) and allowed to adhere overnight. Cells were fixed for 10 min in 4% PFA, followed by 2 M NaCl washes. Detection of FRAP binding was performed as described for the LACE assay.

Isolation of HS from CHO cells

CHO-K1 and pgsF-17 cells were grown to confluency in three T175 flasks (Fisher Scientific). Cells were washed with TES (20 mM Tris, EDTA, and 165 mM NaCl) followed by incubation with 0.25% trypsin for 30 min at 37°C. The cell-trypsin mix was boiled for 30 min and the insoluble fraction was pelleted by centrifugation at 1,500 *g* for 5 min. Protein was precipitated with 6% TCA for 1 h at 0°C and pelleted by centrifugation at 10,000 *g* for 20 min at 4°C. The supernatant was incubated with five volumes of 100% ethanol overnight at −20°C. Precipitated GAG was pelleted by cen-

trifugation at 10,000 *g* for 30 min at 4°C. The resulting pellet was resuspended in sterile water and the total amount of GAG was quantitated using the Alcian blue method (Karlsson and Bjornsson, 2001).

BaF3 proliferation assays

FR2b7, FR2c2, and FR31c cells were added to 96-well flat bottom plates (Fisher) at 10^5 cells/ml in IL-3–deficient media. 10 nM FGF was added and incubated at 37°C for 48 h in the presence or absence of 100 nM porcine intestinal mucosa heparin, 2-ODS heparin, 6-ODS heparin, CHO-K1 HS, or pgsF-17 HS. After 48 h, CellTiter 96 AQueous One Solution reagent (Promega) was added to quantify relative cell numbers using the manufacturer's instructions.

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