

# Role of heparan sulfate as a tissue-specific regulator of FGF-4 and FGF receptor recognition

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**F**GF signaling uses receptor tyrosine kinases that form high-affinity complexes with FGFs and heparan sulfate (HS) proteoglycans at the cell surface. It is hypothesized that assembly of these complexes requires simultaneous recognition of distinct sulfation patterns within the HS chain by FGF and the FGF receptor (FR), suggesting that tissue-specific HS synthesis may regulate FGF signaling. To address this, FGF-2 and FGF-4, and extracellular domain constructs of FR1-IIIc (FR1c) and FR2-IIIc (FR2c), were used to probe for tissue-specific HS in embryonic day 18 mouse embryos. Whereas FGF-2 binds HS ubiquitously, FGF-4 exhibits a restricted pattern, failing to bind HS in the heart

and blood vessels and failing to activate signaling in mouse aortic endothelial cells. This suggests that FGF-4 seeks a specific HS sulfation pattern, distinct from that of FGF-2, which is not expressed in most vascular tissues. Additionally, whereas FR2c binds all FGF-4–HS complexes, FR1c fails to bind FGF-4–HS in most tissues, as well as in Raji-S1 cells expressing syndecan-1. Proliferation assays using BaF3 cells expressing either FR1c or FR2c support these results. This suggests that FGF and FR recognition of specific HS sulfation patterns is critical for the activation of FGF signaling, and that synthesis of these patterns is regulated during embryonic development.

## Introduction

Heparan sulfate (HS)\* is prevalent at the cell surface and in the extracellular matrix of developing organs. Its cell surface distribution is attributed to membrane-intercalated proteoglycans that include the syndecans (Zimmermann and David, 1999), glypicans (Filmus, 2001), CD44(V3) (Bennett et al., 1995; Jackson et al., 1995), and betaglycan (Andres et al., 1989). HS in the matrix is expressed largely on perlecan in basement membranes and the interstitial matrix (Noonan et al., 1991; Iozzo, 1998). HS proteoglycans have diverse functions, ranging from cell adhesion to growth factor signaling, with roles for both the core proteins and HS glycosaminoglycan chains (Bernfield et al., 1999; Park et al., 2000). The most prevalent interactions are via the HS chains for which ligands include matrix components (i.e., collagens, fibronectin, laminins, etc.), growth factors (i.e., FGFs, the EGF family, PDGF-L, hepatocyte growth

factor, vascular endothelial growth factor, etc.), matrix metalloproteinases, lipoproteins and lipases, and viruses (Bernfield et al., 1999).

Binding of growth factors to HS has several potential functions. One established function for FGFs is protection of the growth factors from endogenous proteases (Gospodarowicz and Cheng, 1986). A second is retention of the growth factors at sites of function by the extracellular matrix (Flaumenhaft et al., 1990). Indeed, HS may limit their diffusion and maintain them in active or inactive states, thus generating sites of local activity and morphogenetic boundaries, roles that have been confirmed by emerging genetic studies (Lin et al., 1999; The et al., 1999; Tsuda et al., 1999). Regulation of active or inactive states depends on a third HS function: direct participation of the HS in assembly of the cell surface signaling apparatus. This was first identified in cells deprived of endogenous HS, which curtails FGF binding to its receptor tyrosine kinase (FR) and receptor signaling (Rapraeger et al., 1991; Yayon et al., 1991). This finding has been refined for several members of the FGF family as well as other growth factors, among them hepatocyte growth factor (Sakata et al., 1997; Sergeant et al., 2000), heparan-binding EGF (Kleeff et al., 1998; Paria et al., 1999), and vascular endothelial growth factor (Cohen et al., 1995; Gengrinovitch et al., 1999).

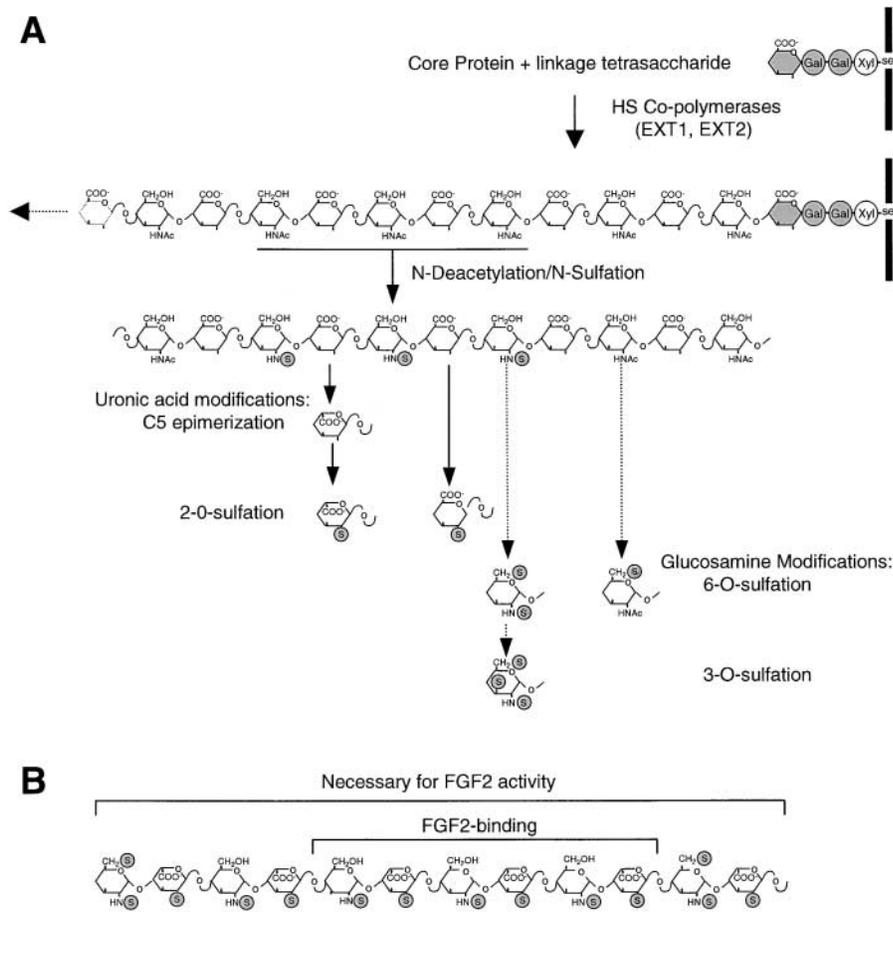
23 members of the FGF family have been identified and each retains an identifiable, although varying, HS-binding

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\*Abbreviations used in this paper: AP, alkaline phosphatase; FGF, fibroblast growth factor; FR, FGF receptor; FRAP, FGF receptor–alkaline phosphatase; HAB, heparin agarose bead; HS, heparan sulfate; MAEC, mouse aortic endothelial cells; PECAM, platelet–endothelial cell adhesion molecule.

Key words: heparan sulfate proteoglycan; fibroblast growth factors; FGF receptors; development; sulfation

**Figure 1. Regulated biosynthesis of heparan sulfate.** (A) HS copolymerases (EXT) synthesize the glycosaminoglycan chain on a linkage tetrasaccharide attached to serine of the proteoglycan core protein. The chain is then modified by a series of concerted enzymatic reactions. The first enzyme to act is an *N*-deacetylase/*N*-sulfotransferase. This enzyme modifies selected sites throughout the chain, although it is unclear how these sites are selected. Regions of the chain that undergo *N*-deacetylation and sulfation of the glucosamine are then targets for further variable modification as shown (modified from Lindahl, 1997) and are interspersed with unmodified regions. The further modifications include epimerization of gluconate to iduronate, 2-*O*-sulfation of the uronic acid or sulfation of the glucosamine in the 6 position. The last family of enzymes to act on the chain are the 3-*O*-sulfotransferases, which act on different sites within the chain depending on which prior modifications have taken place. (B) A postulated HS fragment necessary for FGF-2 binding and activity. The length of the fragment necessary for FGF-2 binding is a hexasaccharide that contains 2-*O*-sulfation of iduronic acid. The length of the fragment necessary for FGF-2 activity is a dodecasaccharide that bears 6-*O*-sulfation of the glucosamine residues. Note that the actual position of the residues within this sequence is not known. Ac, acetyl; circled and shaded S, SO<sub>3</sub>.



domain (Faham et al., 1998; Rapraeger, 2001). These proteins signal through a four-member family of receptor tyrosine kinases (Johnson and Williams, 1993; Szebenyi and Fallon, 1999). The ectodomain of the FR contains three Ig-like domains (D1–D3), in which D2 and D3 mediate FGF binding. The interaction of FGF and D3 is a direct protein–protein interaction using the membrane-proximal half of D3. This region is also subject to splicing variation, which influences FGF binding specificity (Miki et al., 1992; Werner et al., 1992; Ornitz et al., 1996). FGF binding to the D2 region also includes a direct interaction that can vary with FGF type (Chellaiyah et al., 1999), and a second interaction that is mediated by a single HS chain that binds both proteins and stabilizes their association. This utilizes HS binding domains that are present in the FGF and within D2 of the FR (Kan et al., 1993). Multiple models for the FR–FGF–HS interaction have been proposed, attempting to explain how HS promotes binding, oligomerization, and signaling by this monomeric growth factor (Venkataraman et al., 1999; Pellegrini et al., 2000; Schlessinger et al., 2000). In each proposed model, a central question is whether the pattern of sulfation within the HS chain serves to stabilize different FGF and FR pairs, thus regulating the signaling by these growth factors.

It is postulated that HS is encoded with binding specificity by a highly regulated mechanism of synthesis involving sugar epimerization and variable sulfation (Lindahl, 1997;

Lindahl et al., 1998; Guimond and Turnbull, 1999). Synthesis of the HS backbone of alternating glucuronic acid and *N*-acetylglucosamine residues is followed by the action of other enzymes that epimerize and variably modify regions within the chain by sulfation of specific residues (Wei et al., 1993; Kobayashi et al., 1997; Li et al., 1997; Shworak et al., 1997; Habuchi et al., 1998; Lindahl et al., 1998) (Fig. 1 A). Although the number of sites on a single sugar residue that can be sulfated is relatively few, the potential variation within a span of many saccharides increases geometrically with each subsequent disaccharide added, and leads to the formation of discrete domains within the HS chain. In fact, *in vitro* experiments with a library of HS saccharides generated by enzymatic or chemical cleavage has demonstrated that FGF binding to HS and signaling depends upon such block sequences of variable sulfation that arise during synthesis of the glycosaminoglycan chain (Turnbull et al., 1992; Guimond et al., 1993; Pye et al., 1998) (Fig. 1 B). However, an important question is whether specific HS binding structures are expressed in a distinct pattern *in vivo*. If specific binding domains are indeed present within different tissues, the next question is whether they are selectively recognized by specific FGFs, and whether the recognition of these FGF–HS complexes by individual FRs is also determined by the HS to which the FGF is bound.

To directly look for tissue-specific HS expression patterns that may regulate growth factor activity, we have used FGF-2

and FGF-4 as probes to detect HS in frozen sections of the developing mouse embryo. In addition, the ability of tissue-specific HS to assimilate these FGFs into a complex with an FR was tested with two soluble FR constructs. FGF-2 and FGF-4 were chosen because they reportedly bind to the same signaling receptors, act on a wide variety of cell types, and have similar mitogenic activities using reporter cells (Ornitz et al., 1996; Szebenyi and Fallon, 1999). The two soluble receptor probes were FGF receptor 1-IIIc variant (FR1c) and FGF receptor 2-IIIc variant (FR2c), consisting of the ectodomains of these FRs expressed as fusion proteins with human placental alkaline phosphatase (AP). These receptor isoforms recognize both FGF-2 and FGF-4 when bound to heparin, a highly sulfated form of HS. The results of using these probes on endogenous HS in the embryonic day 18 (E18) mouse demonstrate that although FGF-2 and FGF-4 bind to HS in many of the same tissues, FGF-4 does not recognize HS in the heart and major blood vessels, suggesting that (a) FGF-2 and FGF-4 recognize different HS chains, or different sulfation patterns within the same HS chain, and (b) the sulfation pattern recognized by FGF-4 is not expressed in most vascular tissues. Second, although FR1c and FR2c bind FGF-2 and FGF-4 equally in the presence of heparin, endogenous HS supports the binding of FGF4 to FR1c only in rare cases, whereas HS supports the binding of FGF-4 to FR2c in all sites examined. This demonstrates not only that HS is expressed in a cell- and tissue-specific manner, but that the specific synthesis of HS may be a critical regulator of HS-binding growth factors.

## Results

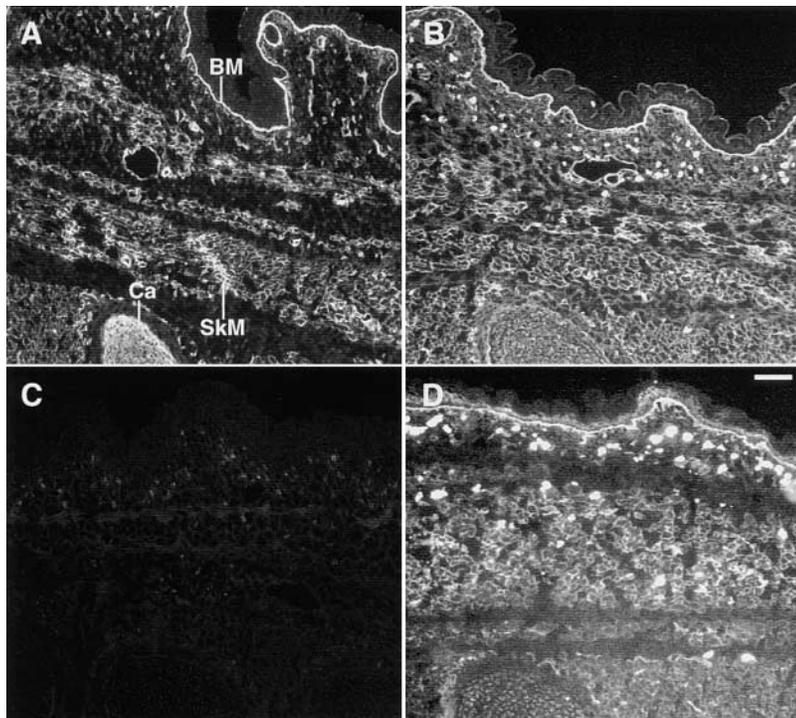
### FGF-2 and FGF-4 bind HS in E18 mouse skin

FGF-2 and FGF-4, like all members of the FGF family, bind heparin. In addition, when bound by heparin, both

FGFs activate FR1c and FR2c. However, because HS has a more variable sulfation pattern than heparin, it is not clear that HS expressed by all cell types would promote these interactions. To examine the possibility that HS is synthesized with a tissue-specific structure, exogenous FGF-2 and FGF-4 were used as probes for specific HS in tissue sections from E18 mouse embryos. Total HS was mapped with mAb3G10, which recognizes the residual HS stub on endogenous core proteins after digestion with heparitinase. Antibody staining demonstrates that HS is prevalent throughout the epidermis and dermis of E18 mouse skin, exhibiting prominent staining in the epithelial basement membrane, in skeletal muscle, and in cartilage of the developing rib (Fig. 2 A). FGF-2 staining mirrors that of HS, suggesting that it binds HS ubiquitously (Fig. 2 B). Binding is seen at cell surfaces and also in basement membranes, as well as elsewhere in the matrix. Importantly, binding to the tissue sections is completely blocked by HS degrading enzymes (Fig. 2 C), suggesting that the binding of FGF-2 is completely HS dependent. Detection of FGF-4 in the skin yields similar results. FGF-4 binds ubiquitously to HS (Fig. 2 D) and, like FGF-2, fails to bind following pretreatment with heparitinase (unpublished data).

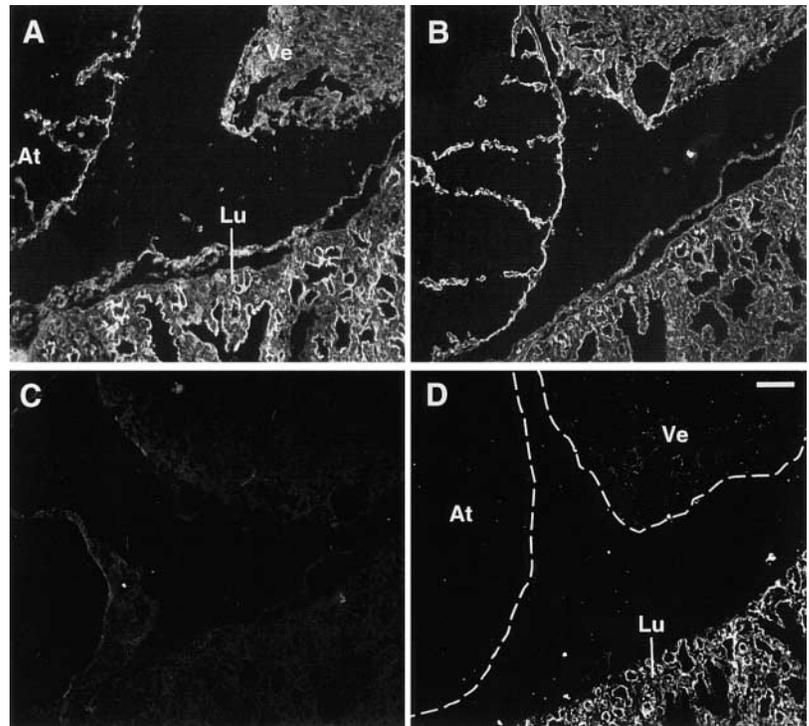
### FGF-4 fails to bind HS in E18 mouse heart

Despite the similarities in FGF-2 and FGF-4 recognition of HS in the mouse skin, other sites in the mouse display strikingly different binding patterns. One example is HS in the E18 mouse heart. Staining with mAb3G10 identifies HS throughout the atrium and ventricle of the heart, as well as in neighboring lung tissue (Fig. 3 A). As seen in the skin, the FGF-2 binding pattern is identical to that of the HS distribution (Fig. 3 B), and is completely dependent on HS (Fig. 3 C). However, FGF-4, which binds strongly to HS in the



**Figure 2. FGF-2 and FGF-4 bind specifically to endogenous HS in E18 mouse skin.** The area shown is a section of skin in an E18 stage mouse embryo. (A) Total HS distribution is detected by mAb3G10 following treatment of the section with the heparin lyase I and heparin lyase III (heparitinase treatment). (B) Exogenous FGF-2 binding is detected with Ab DE6 after incubation with 30nM FGF-2. (C) FGF-2 binding following pretreatment with heparitinase. (D) Exogenous FGF-4 binding is detected with Ab AF235 after incubation with 30 nM FGF-4. BM, basement membrane; Ca, cartilage; SkM, skeletal muscle. Bar, 100  $\mu$ m.

**Figure 3. FGF-2 and FGF-4 binding to HS in the E18 mouse heart and lung.** A section of the atrium and ventricle of the E18 mouse heart, as well as neighboring lung tissue is shown. Treatment and detection are as described in Fig. 2. (A) Total HS detected with mAb 3G10; (B) binding of FGF-2; (C) binding of FGF-2 following heparitinase treatment; (D) binding of FGF-4. Dashed lines represent the borders of the atrium and ventricle, which do not stain in (D). At, atrium; Ve, ventricle; Lu, lung. Bar, 100  $\mu$ m.



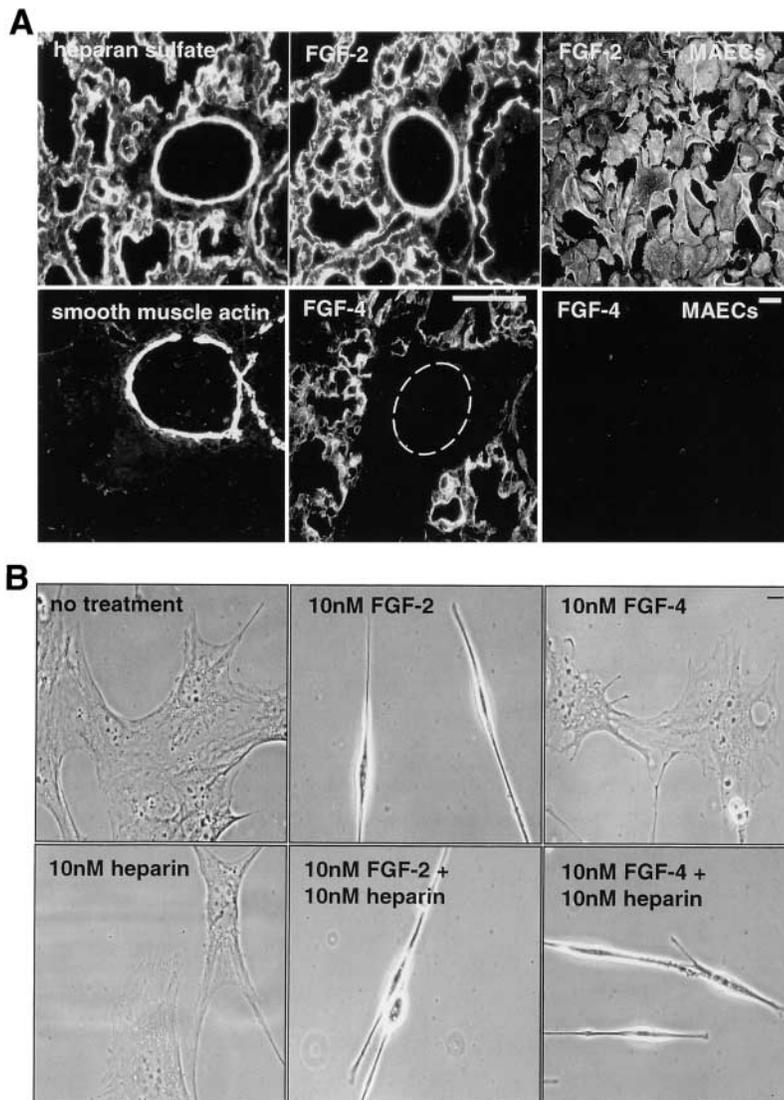
lung, fails to recognize HS in either the atrium or the ventricle of the heart (Fig. 3 D).

Examination of the lung provides further insight into the FGF binding specificity. In the lung, mAb3G10 identifies HS that is prevalent not only within lung tissue, but also within blood vessels (Fig. 4 A). A major blood vessel is identified by smooth muscle actin staining. Although FGF-2 binds HS in both lung tissue and the vessel, FGF-4 binds HS in the lung tissue, but fails to bind the blood vessel HS. This suggests that endothelial and smooth muscle cells lining the vessel express HS that contains a specific sulfation pattern that is recognized by FGF-2, but not by FGF-4. This was tested directly by examination of the binding of either FGF-2 or FGF-4 to HS on cultured mouse aortic endothelial cells.

Whereas FGF-2 binds HS on the endothelial cells, FGF-4 binding is negative. To confirm that the apparent failure of FGF-4 to bind to HS on mouse aortic endothelial cells (MAECs) correlates to a lack of activity, cells were treated with either FGF-2 or FGF-4 in the absence or presence of heparin (Fig. 4 B). Importantly, MAECs have been shown to express FR2c, an FR isoform that is thought to be equally activated by FGF-2 and FGF-4 (Ornitz et al., 1996; Bastaki et al., 1997). In the absence of any treatment, MAECs adopt a highly spread morphology. Treatment with FGF-2 stimulates these cells to drastically change their morphology, adopting a spindle-like, polar morphology and likely reflects FGF-induced motility. In contrast, FGF-4 treatment stimulates no morphology change in these cells. However, treatment with

**Table I. FGF and FR recognition of tissue-specific HS in E18 mouse embryos**

HS site	Probe					
	FGF-2	FGF-2 +		FGF-4	FGF-4 +	
		FR1cAP	FR2cAP		FR1cAP	FR2cAP
Skin	+	+	+	+	-	+
Skeletal muscle	+	+	+	+	-	+
Mast cells	+	+	+	+	+	+
Gut	+	+	+	+	-	+
Rib	+	+	+	+	-	+
Kidney	+	+	+	+	+	+
Lung	+	+	+	+	-	+
Liver	+	+	+	+	+	+
Submandibular gland	+	+	+	+	-	+
Adrenal gland	+	+	+	+	-	+
Atrium	+	+	+	-	-	-
Ventricle	+	+	+	-	-	-
Artery	+	+	+	-	-	-
Vein	+	+	+	-	-	-
Brain capillary	+	+	+	+	-	+



**Figure 4. FGF-2 and FGF-4 binding to endothelial HS and signaling in cultured endothelial cells.** (A) A region of the E18 mouse lung containing a large blood vessel is shown, with FGF treatments as described in Fig. 2. Top panels (from left to right): mAb 3G10 detection of total HS; binding of FGF-2 to tissue; FGF-2 binding to cultured mouse aortic endothelial cells (MAECs). Bottom panels (from left to right): staining for smooth muscle actin; binding of FGF-4; FGF-4 binding to MAECs. Dashed circle denotes the border of a large artery within the lung, to which FGF-4 fails to bind. (B) Assessment of MAEC morphology after FGF treatment. Top panels (from left to right): no treatment; 10 nM FGF-2; 10 nM FGF-4. Bottom panels (from left to right): 10 nM heparin; 10 nM FGF-2 + 10 nM heparin; 10 nM FGF-4 + 10 nM heparin. Bars: (A) Bottom middle panel, 100  $\mu$ m; Bottom right panel, 100  $\mu$ m; (B) Top right panel, 20  $\mu$ m.

heparin, which has no effect alone, causes MAECs to adopt this spindle-like morphology in the presence of either FGF-2 or FGF-4. These data are consistent with previously published results regarding the response of endothelial cells to FGF-2 and FGF-4 (Delli-Bovi et al., 1988; Dell’Era et al., 2001), and suggest that the fine structure of HS on these cells regulates their response to different FGF family members.

Although FGF-4 fails to recognize HS in both the heart and large blood vessels, there is vascular HS that is recognized by FGF-4. In E16 mouse embryos, mAb3G10 staining confirms the presence of HS in capillaries and the choroid plexus within the brain (Fig. 5 A). The capillaries are identified by staining for platelet–endothelial cell adhesion molecule (PECAM)1 (unpublished data). FGF-2 binds HS in the capillaries and in the choroid plexus (Fig. 5 B). FGF-4 fails to bind HS in the choroid plexus, similar to the difference seen between FGF-2 and FGF-4 binding in the heart. However, FGF-4 does recognize the HS in the brain capillaries with apparently equal intensity as the FGF-2 binding (Fig. 5 D).

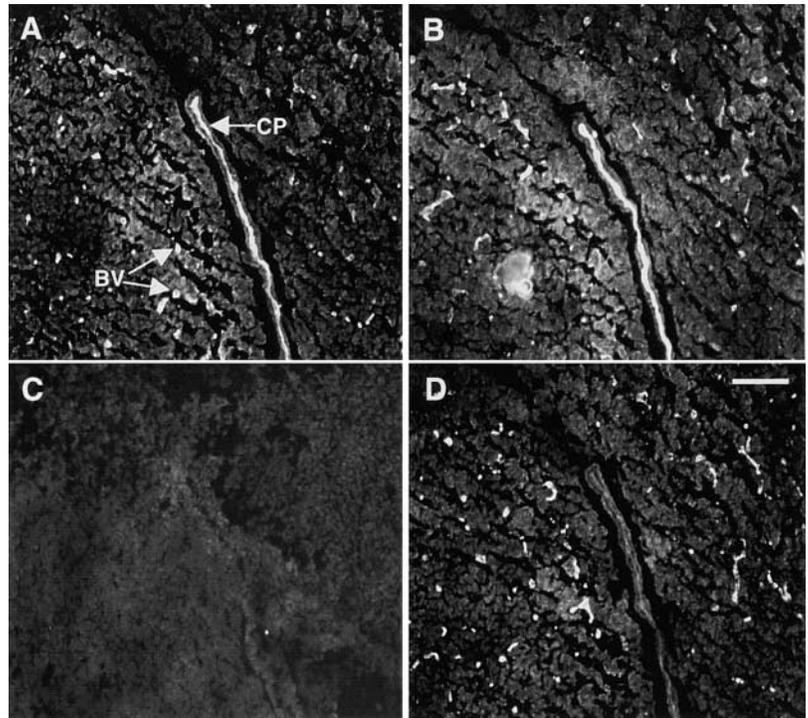
The binding of FGF-2 and FGF-4 to major HS sites in the E18 mouse embryo is summarized in Table I. FGF-2 binds to HS in all sites examined, suggesting that the sulfa-

tion pattern necessary for its binding is ubiquitous. Interestingly, FGF-4 binds HS in many of the same organs as FGF-2, including lung, liver, and kidney. FGF-4 also binds HS in glandular structures, such as the adrenal gland as well as the submandibular gland. As described above, the most striking results are in the vascular system, where FGF-4 fails to bind HS in both the heart and in large blood vessels, although it does recognize HS in brain capillaries.

#### FR1cAP and FR2cAP bind equally to FGF-2 and FGF-4 immobilized on heparin

Regulation of FGF signaling by HS is dependent not only on FGF binding to sites within the HS chain, but also on simultaneous FR recognition of the HS. The specificity required for receptor recognition is largely unknown, although it has been shown that a library of HS decasaccharides can differentially activate FGF signaling in cells engineered to express specific FRs (Guimond and Turnbull, 1999). It is possible that the HS specificity required for FGF binding is also satisfactory for receptor binding, particularly if the binding is stabilized by FGF–FR interactions. Alternatively, receptor binding may be highly dependent on specific sulfa-

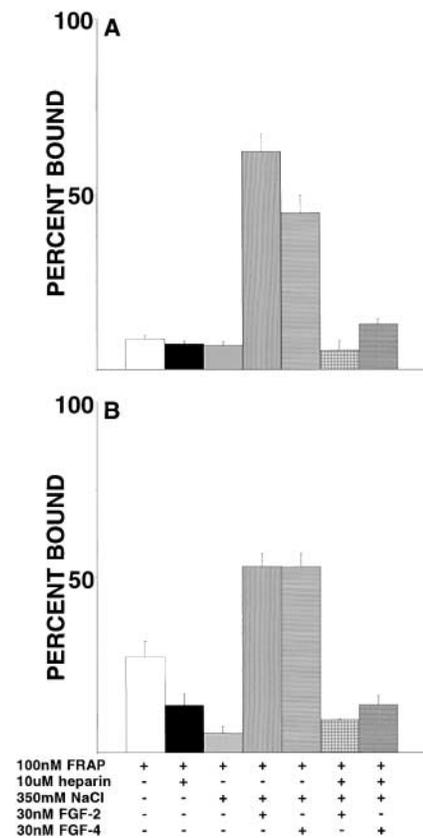
**Figure 5. FGF-4 binds capillary HS in the brain.** Serial sections of E16 mouse brain, treated with FGFs as in Fig. 2. (A) Total HS localized by mAb3G10. (B) Binding of FGF-2. (C) Binding of FGF-2 after heparitinase treatment. (D) Binding of FGF-4. BV, blood vessels; CP, choroid plexus. Blood vessels are identified by staining with anti-PECAM-1 Ab (unpublished data). Bar, 100  $\mu$ m.



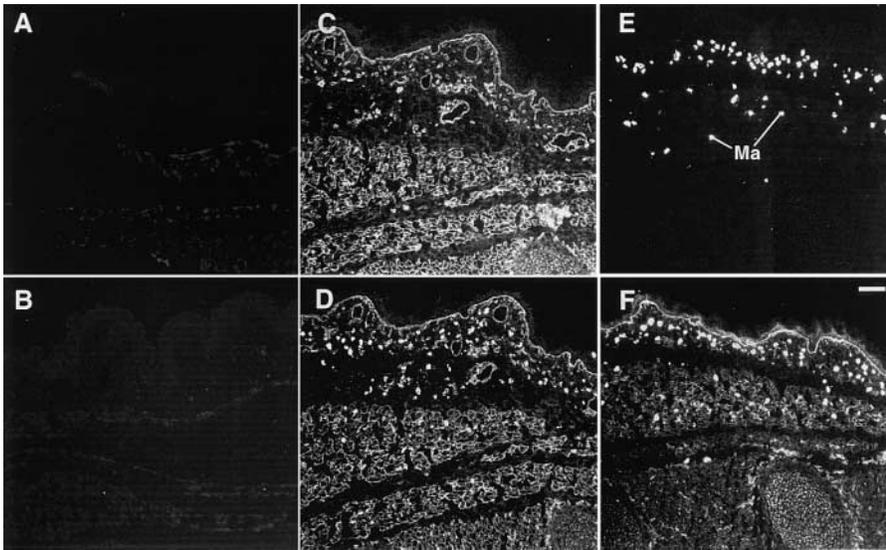
tion patterns such that FGFs will only be recognized by the receptor when bound to sites containing HS sequences that are specific for both the FGF and the FR.

To test these possibilities, soluble extracellular ligand binding domains of FR1c and FR2c fused to human placental alkaline phosphatase (FR1cAP and FR2cAP, respectively) were used as probes to address receptor binding to the FGF-HS complexes assembled on the tissue sections. Both FR1c and FR2c are strongly activated by complexes of FGF-2-heparin and FGF-4-heparin, and so are ideal receptors to use as probes for the detection of tissue-specific HS. Both FR1cAP and FR2cAP were expressed in COS-7 cells and purified from COS-7-conditioned medium. Because each FR contains an HS binding domain, receptor constructs were subjected to 1-M NaCl washes during purification to remove any endogenous HS that may have bound the FR. Comparison of constructs purified with or without the 1-M NaCl wash showed no difference in the ability of either FRAP construct to bind to heparin or HS, suggesting that either no endogenous HS copurified with the receptors, or that a sufficiently low amount of HS copurified such that no differences could be detected (unpublished data).

To confirm that the receptor constructs are functional, each receptor was incubated with heparin agarose beads (HABs) in the presence or absence of either FGF-2 or FGF-4. In the absence of FGF, FR1cAP fails to bind HABs (Fig. 6 A, white bar), although FR2cAP does display some binding (Fig. 6 B, white bar). The binding of FR2cAP to HABs is specific, as it is competed with excess soluble heparin (Fig. 6 B, black bar). Additionally, FR2cAP binding to heparin can also be disrupted by washing the beads with 0.35 M NaCl (Fig. 6 B, gray bar). In contrast, in the presence of either FGF-2 or FGF-4, FR1cAP and FR2cAP bind strongly (Fig. 6, A and B, vertical and horizontal lined bars), and this is not disrupted by



**Figure 6. FR1cAP and FR2cAP binding to FGF-2-heparin and FGF-4-heparin on agarose beads.** Percent of FRAP bound to HABs: in the absence of FGF; after incubation with excess soluble heparin; after 0.35 M NaCl wash; after 0.35 M NaCl wash in the presence of FGF-2 or FGF-4; after incubation with excess soluble heparin in the presence of FGF-2 or FGF-4. (A) FR1cAP; (B) FR2cAP.



**Figure 7. FR1cAP and FR2cAP binding to FGF-2–HS complexes and FGF-4–HS complexes in E18 mouse skin.** A region of mouse skin (top), dermis, and body wall is shown. The sections were incubated either with no FGF (A and B), 30 nM FGF-2 (C and D), or 30 nM FGF-4 (E and F). After washing, the sections were incubated with 100 nM FR1cAP (A, C, and E) or 100 nM FR2cAP (B, D, and F). Bound FRAP is observed using anti-AP antibody. Ma, mast cells. Bar, 100  $\mu$ m.

a 0.35-M NaCl wash. This is consistent with previously published results (Ornitz et al., 1992). As with the binding of FR2cAP alone, formation of a ternary complex of FGF–HAB–FRAP is abrogated by incubation with excess soluble heparin (Fig. 6, A and B, cross-hatched and brick bars).

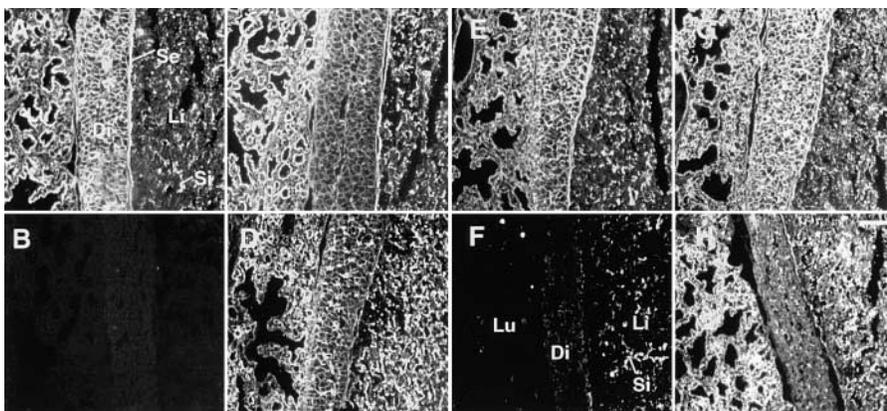
#### FR1cAP fails to recognize FGF-4–HS complexes in E18 mouse skin

To determine whether FR1cAP and FR2cAP bind FGF-2 and FGF-4 equally when endogenous HS participates rather than heparin, the soluble receptors were used to probe E18 mouse skin, where both FGF-2 and FGF-4 bind equally to endogenous HS (compare Fig. 7 with Fig. 2). FR1cAP and FR2cAP were incubated with tissue sections in either the absence or presence of FGF-2 or FGF-4. In the absence of FGF, neither receptor binds to HS in E18 mouse skin tissue sections (Fig. 7, A and B). In the presence of FGF-2, both FR1cAP and FR2cAP bind FGF-2–HS in an identical pattern that corresponds to the distribution of FGF-2 (Fig. 7, C and D). Again, this binding is dependent on the presence of FGF-2–HS complexes, as pretreatment with heparitinase completely abrogates receptor binding (unpublished data). Interestingly, in the presence of FGF-4, FR1cAP fails to bind any FGF-4–HS complexes in the skin (Fig. 7 E), whereas FR2cAP appears to bind all FGF-4–HS complexes

in the section (Fig. 7 F). As an important internal control, FR1cAP does not recognize FGF-4–heparin complexes as shown by the bright punctate staining of mast cells, which contain heparin in  $\alpha$ -granules (Fig. 7 E). This result suggests that whereas FR1cAP does recognize FGF-4 in the context of highly sulfated heparin, HS in the skin lacks the appropriate sulfation sequence necessary for the recognition of FGF-4 by FR1cAP. Additionally, these results suggest that HS in the skin does contain the appropriate sulfation sequence necessary for FR1cAP recognition of FGF-2 and FR2cAP recognition of both FGF-2 and FGF-4.

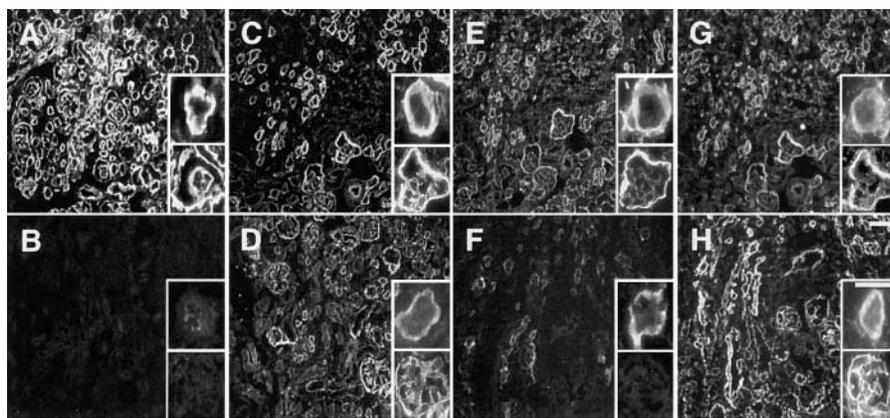
#### FR1cAP recognizes FGF-4–HS in the E18 mouse liver and kidney

Whereas FR1cAP fails to recognize FGF-4 bound to HS in the E18 mouse skin, there are specific sites where FR1cAP does recognize FGF-4–HS complexes. One such site is in the E18 mouse liver. In a section containing the lung, diaphragm, and liver, 3G10 staining identifies the presence of HS at cell surfaces including lung epithelia, muscle of the diaphragm, and hepatocytes within the liver (Fig. 8 A). In addition, HS is prominent in the basement membrane of the lung, and serosal lining of the liver, as well as the lining of the liver sinusoids. Exogenous FGF-2 recognizes HS in all of these tissues (Fig. 8 C). However, whereas FGF-4 binds HS



**Figure 8. FR1cAP and FR2cAP binding to FGF–HS complexes in E18 mouse liver.** A section of E18 mouse lung (Lu), diaphragm (Di) and liver (Li) is shown. Sections were incubated with 30 nM FGF-2 (B, C, E, and G) or 30 nM FGF-4 (D, F, and H). After washing, sections were incubated with 100 nM FR1cAP (E and F) or 100 nM FR2cAP (G and H). Antibodies were used to detect total HS (A); FGF-2 binding after prior heparitinase treatment (B); bound FGF-2 (C); bound FGF-4 (D); bound FR1cAP (E and F); and bound FR2cAP (G and H). Se, serosal lining of liver; Si, lining of liver sinusoids. Bar, 100  $\mu$ m.

**Figure 9. FR1cAP binds FGF-4–HS in renal tubules of E18 mouse kidney.** A section of E18 mouse kidney is shown. Higher magnification views of a glomerulus (lower inset) and a renal tubule (upper inset) are also shown. Sections are incubated with 30 nM FGF-2 (B, C, E, and G) or 30 nM FGF-4 (D, F, and H). Following washing, sections were incubated with 100 nM FR1cAP (E and F) or 100 nM FR2cAP (G and H). Total HS (A), FGF-2 binding following heparitinase treatment (B), FGF-2 (C), FGF-4 (D) FR1cAP (E and F) and FR2cAP (G and H) are visualized by immunostaining. Bars: (H) 100  $\mu$ m; (inset) 100  $\mu$ m.



in the lung epithelium and diaphragm, it binds only weakly to HS in the serosa of the liver, although it does bind strongly to the lining of the liver sinusoids (Fig. 8 D). In contrast, both FR1cAP and FR2cAP bind FGF-2–HS throughout the lung, liver, and diaphragm (Fig. 8, E and G). Although FR1cAP appears to recognize FGF-2 in the diaphragm more avidly than FGF-2 bound to HS in the lung, this is not a consistent result and is likely a source of experimental variation. FR2cAP also binds FGF-4–HS in the lung, diaphragm, and liver (Fig. 8 H), preferentially binding to FGF-4–HS complexes in the lung rather than the diaphragm. However, FR1cAP fails to bind FGF-4–HS in either the lung or in the diaphragm (Fig. 8 F). Nonetheless, FR1cAP is able to recognize FGF-4–HS in the lining of the liver sinusoids. Although this binding is not as strong as that seen with FR1cAP binding to FGF-2–HS, it does suggest that there is a subset of HS in the liver that promotes FGF-4 binding to FR1cAP.

A second site where FR1cAP recognizes FGF-4 bound to endogenous HS is in the E18 mouse kidney. HS is identified throughout the kidney, including prominent staining in both the glomeruli and renal tubules (Fig. 9 A, insets). Both FGF-2 and -4 bind HS strongly in the kidney (Fig. 9, C and D), binding that is abolished by prior heparitinase treatment (shown for FGF-2 in Fig. 9 B). FR1cAP recognizes FGF-2–HS throughout the kidney (Fig. 9 E), as does FR2cAP (Fig. 9 G). FR2cAP also recognizes FGF-4–HS in the kidney, including strong staining in both the glomeruli and renal tubules (Fig. 9H). However, FR1cAP fails to bind FGF-4–HS in the glomeruli of the kidney, although it does recognize FGF-4–HS in the renal tubules (Fig. 9 F). This suggests that there are multiple HS sequences present in different structures within the kidney, and that a specific HS sulfation sequence is present in renal tubules, but lacking in glomeruli, that is necessary for FR1cAP recognition of FGF-4.

FR1cAP and FR2cAP recognition of FGF-2 and FGF-4 when bound to endogenous HS is summarized in Table I. FR1cAP binds FGF-2–HS throughout the E18 embryo. In contrast, FR1cAP fails to recognize the majority of FGF-4–HS complexes. The two major exceptions to this are the recognition of FGF-4–HS in the lining of the sinusoids of the liver and within renal tubules of the kidney. These results, when taken into consideration with the ability of FR1cAP to recognize FGF-4–heparin in mast cells, suggest that a spe-

cific and rare HS sulfation sequence is necessary for FR1c recognition of FGF-4.

### HS regulation of FR1c and FR2c signaling

To confirm that the binding of soluble FRAPs to FGF–HS complexes on the frozen tissue sections recapitulates the mechanism by which an active FGF–HS–FGFR signaling complex is assembled, cell proliferation assays were performed with BaF3 cells expressing either FR1c or FR2c (FR1c11 and FR2c2 cells, respectively). The parental BaF3 cells are a lymphoid cell line that is negative for both HS and FGF receptor expression. These cells normally require interleukin (IL)3 for survival; however, cells expressing FR constructs overcome this requirement and survive and proliferate when grown in the presence of the appropriate FGF together with heparin. Both FR1c11 cells and FR2c2 cells have been previously shown to proliferate equally in response to treatment with either FGF-2 or FGF-4 in the presence of heparin (Ornitz et al., 1996). The response to FGF-4, either in the presence or absence of heparin is also demonstrated here, indicating that this FGF activates either FR1c or FR2c in the presence of this glycosaminoglycan (Fig. 10, B and C).

To provide a source of HS that has the characteristics of the majority of endogenous HS, which promotes binding of FR1cAP to FGF-2 but not to FGF-4, the binding of these FGFs and FRAPs were tested on Raji-S1 cells, a human lymphoblastoid cell line that has been transfected with the cDNA for mouse syndecan-1 (Lebakken and Rapraeger, 1996). Indeed, the HS expressed on the syndecan-1 of these cells contains a sulfation pattern similar to that seen in the majority of the mouse embryo, namely, binding FGF-2 and FGF-4 and promoting binding of FR2c AP to both growth factors, and promoting binding of FR1c AP to FGF-2 but not FGF-4 (Fig. 10 A).

To test the ability of these receptors to be activated by the FGF–HS complexes on the Raji-S1 cells, the BaF3 cells expressing either FR1c or FR2c were cultured with FGF-2 or FGF-4 on fixed monolayers of Raji-S1 cells. Similar to our prior published work (Filla et al., 1998), the FR1c11 cells cultured on fixed monolayers of Raji-S1 cells in the presence of FGF-2 utilize the HS on the Raji cells to bind and respond to the FGF (Fig. 10 B). In contrast, FR1c11 cells do not respond to FGF-4 when grown on a fixed monolayer of

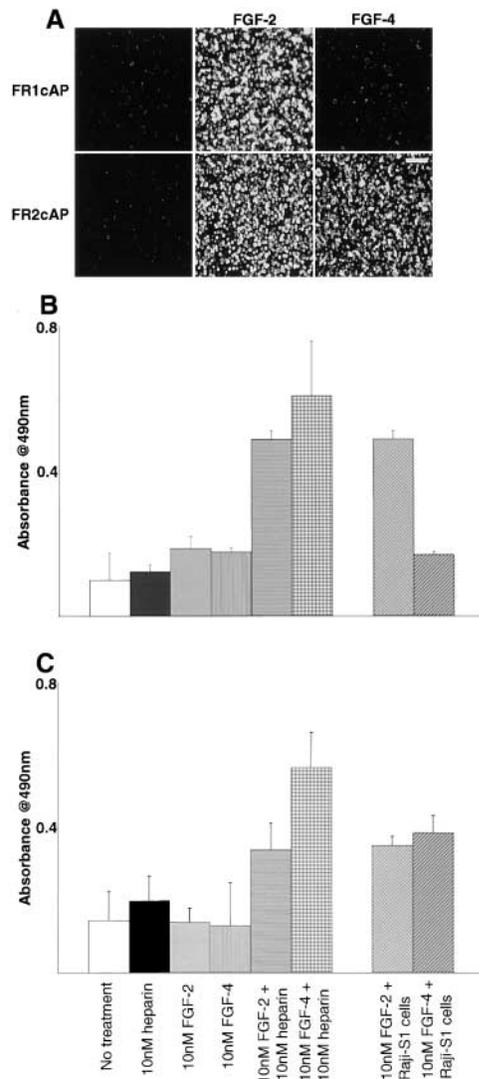


Figure 10. **Regulation of binding and signaling of FR1c and FR2c by FGF bound to HS on Raji-S1 cells.** (A) Fixed Raji-S1 cells are incubated with either with no FGF, 30 nM FGF-2 or 30 nM FGF-4, followed by 100 nM FR1cAP or FR2cAP. Bound receptor is detected with anti-AP antibody. (B) BaF3 cells expressing FR1c (FR1c11 cells) are either incubated in culture medium with no treatment, or incubated in medium with 10 nM heparin, 10 nM FGF-2 or FGF-4, or 10 nM FGF-2 or FGF-4 + heparin. Alternatively, the FR1c11 cells are cultured on a fixed monolayer of Raji-S1 cells in the presence of 10 nM FGF-2 or 10 nM FGF-4. After 3 d, relative cell number is assessed ( $A_{490}$ ). (C) BaF3 cells expressing FR2c (FR2c2 cells) are treated as in B. Bar, 200  $\mu$ m.

Raji-S1 cells, confirming the failure of FR1c to recognize FGF-4 bound to this HS. FR2c2 cells also confirm the results seen using the FGFs and FRAP receptor probes in situ (Fig. 10 C). In contrast to the FR1c11 cells, the FR2c2 cells respond to both FGF-2 and FGF-4 when grown on a fixed monolayer of the Raji-S1 cells shown to express HS that promotes binding of FGF-4 to FR2c.

## Discussion

A central question in the field of proteoglycan biology has been whether the complex sequence of modifications that

occur during glycosaminoglycan synthesis serves a functional role in vivo, particularly in the tissue specific regulation of growth factor signaling. The integral role of heparan sulfate as a component of the FGF signaling complex, where it assembles with the FGF and the FR, suggests that the generation of specific sulfation patterns within the chain by specific cell types may regulate assembly of FGF family members with their receptors. To address the question of whether tissue-specific HS regulates FGF binding, exogenous FGF-2 and FGF-4 were used to probe and identify FGF-specific HS in the developing mouse embryo. Although both FGF-2 and FGF-4 recognize heparin in vitro (and in mast cells in vivo), distinct differences exist in the ability of FGF-2 versus FGF-4 to bind HS in vivo. In the E18 mouse, FGF-2 recognizes HS in a ubiquitous manner, suggesting that essentially all HS binds this growth factor. In contrast, whereas FGF-4 binds HS in many of the same sites as FGF-2, there are other sites where FGF-4 fails to bind. A stark contrast is seen in the vascular system where FGF-4 fails to bind to HS in the heart and large blood vessels, as well as to aortic endothelial cells in culture. Importantly, the failure of FGF-4 to bind HS on these cells correlates with a failure of FGF-4 to stimulate a response in these cells. However, a response can be stimulated, when heparin is added, suggesting that the correct FRs are indeed expressed on these cells, but that the endogenous HS is inappropriate for FGF to bind and signal. This result is surprising, as FGF-4, also known as k-FGF (Delli-Bovi et al., 1988) or *hst* (Miyagawa et al., 1988), was originally identified as an angiogenic factor with activity toward endothelial cells. However, these studies typically added heparin along with the FGF to achieve activity (Delli-Bovi et al., 1988; Yoshida et al., 1994; Dell'Era et al., 2001). Interestingly, FGF-4 binding to endothelial HS seems to vary among blood vessels of different origin, as FGF-4 does bind to HS in capillaries of the brain. A more detailed investigation will be necessary to determine whether this extends to all capillaries, or whether this is a function of the tissue type, as the phenotype of endothelial cells in various tissues is clearly affected by the cadre of neighboring cells (Aird et al., 1997).

It is also surprising that FGF-4 fails to bind in the heart because of its role in early heart development. There is nothing known about FGF-specific HS expression during heart development, although it is clear that FGF induction of precardiac differentiation in vitro requires HS (Zhu et al., 1996). Heart development from precardiac mesenchyme is initiated before gastrulation by paracrine signaling originating in the endoderm and acting on the adjacent mesodermal layer. This has been studied mostly in the chick embryo in which the underlying endoderm at stage 5 expresses FGF-4, along with FGF-1 and FGF-2. Any of these FGFs will induce the proliferation and differentiation of cardiac myocytes from the precardiac mesenchyme (Lough et al., 1996; Zhu et al., 1996). These same FGFs reappear later during heart chamber formation, where they are expressed in the myocardium with autocrine roles in cardiomyocyte proliferation and differentiation (Zhu et al., 1996). Expression of these FGFs in the heart is subsequently lost at later stages of development. Interestingly, FGF expression in the chick is largely paralleled by expression of FR1, which peaks at stage

24, the stage when heart chamber formation is completed, but persists until day 7 (Sugi et al., 1995). Expression then declines in the ventricle but persists in the atrium. Importantly, these stages of FGF-4 expression in the chick compare with E9–E11 in the mouse, which precede the mouse E18 stage examined here. Because FGF-4 fails to bind the heart HS and FR1c recognizes it only rarely when bound elsewhere in the E18 embryo, it suggests that the HS structure may change with development. This is indeed suggested by our preliminary data (unpublished data).

These results suggest that specific differences exist in HS of both the heart and large blood vessels such that FGF-2, but not FGF-4, is recognized. The ability of FGF-2 to bind to most, if not all HS, indicates either that the FGF-2 binding motif in HS is a common HS sequence that is present in all tissues, or that FGF-2 is able to recognize multiple HS sulfation patterns, such that binding is not dramatically affected by variations in HS structure. When examined in detail using isolated heparin or HS fragments, it has been shown that FGF-2 binding is dependent on the minimum of a pentasaccharide containing 2-*O*-sulfation (Turnbull et al., 1992; Guimond et al., 1993; Maccarana et al., 1993) (Fig. 1 B). Thus, the presence of 2-*O*-sulfation may be sufficient for FGF-2 binding, regardless of what other sulfate groups are present.

The failure of FGF-4 to bind in the vascular tissue suggests that the binding motif for FGF-4 is different from that of FGF-2, and that this motif is lacking in the heart and major blood vessels. Although there are fewer data regarding the HS binding requirements of FGF-4 than FGF-2, previous studies have suggested that binding of this growth factor is dependent on HS containing a high content of *N*-sulfo-glucosamine-bearing 6-*O*-sulfate groups (Guimond et al., 1993). This supports the findings here that FGF-2 and FGF-4 recognize different sites; however, these prior experiments, which are aimed primarily at specific types of sulfation (i.e., 2-*O*-sulfation, or 6-*O*-sulfation) rather than specific motifs within the HS chain, provide insufficient information on what the specific motifs might be.

Overall, these results suggest that the HS in the walls of vascular elements has very different FGF binding capabilities, which may have far-reaching implications during vascular development and tumor-mediated angiogenesis, where FGFs are known to play an important role (Slavin, 1995; Beckner, 1999). Additionally, the finding that tissue-specific HS regulates FGF binding is likely to have a major impact not only on the 23 FGFs, but also on other HS-binding growth factors, including BMPs, wnts, and hedgehogs, among others (Bernfield et al., 1999).

The ability of FGFs to bind tissue-specific HS fulfills only part of the requirement necessary for activation of FGF signaling, however, as FRs must also recognize specific HS structures in order to bind to and be activated by a particular FGF. Previous studies have shown that FGF-2 activity via FR1c requires a dodecasaccharide (twice the length necessary for binding alone) bearing glucosaminyl-6-*O*-sulfates in addition to the iduronosyl-2-*O*-sulfates necessary for FGF-2 binding (Guimond et al., 1993; Pye et al., 1998; Turnbull et al., 1992) (Fig. 1 B). This additional length and sulfation requirement represents a second level of HS specificity that is likely to be

important for assembly with FRs leading to signaling. Indeed, heparin depleted of 6-*O*-sulfates will bind FGF-2 essentially as well as native heparin or HS, but will inhibit the growth factor by failing to assemble with the FR (Guimond et al., 1993). Importantly, recent evidence suggests that it is not merely the presence of 6-*O*-sulfates that is critical for signaling, but also the location of the 6-*O*-sulfates on the HS chain that plays a critical role in FR activation (Guimond and Turnbull, 1999).

In the current study, FR1c recognizes FGF-2–HS complexes throughout the E18 mouse embryo; this recognition is duplicated by FR2c. Given these data alone, it would be tempting to conclude that HS serves as nothing more than a nonspecific partner for the FGF and receptor. However, when taken in the context of the inability of FR1c to recognize FGF-4–HS in the vast majority of sites within the embryo, a different story emerges. The fact that FR1c recognizes FGF-4–heparin both *in vitro* and *in vivo*, but fails to recognize FGF-4–HS at most sites suggests that FRs do indeed require specific HS sulfation sequences in order to recognize a specific FGF. Additionally, the data suggest that the HS sequence necessary for FGF recognition differs between FRs, as FR2c does recognize FGF-4–HS throughout the embryo. Indeed, one wonders if FR2c and FR1c are binding exactly the same sites even when they bind to the FGF-2–HS complexes. It is entirely possible that these are actually distinct HS chains, or at least distinct sites on HS chains.

Previous studies provide at least partial explanations for the two tissues, namely the liver and the kidney, where FR1c does recognize FGF-4. In the liver, the structure of HS has been characterized as being highly sulfated, and in fact, heparin-like (Lyon et al., 1994). As a result, it is likely that the rare sulfation sequence necessary for FR1c recognition of FGF-4, which exists in heparin, also exists on the heparin-like HS chains present in this site. In the kidney, it has been shown that the HS is heterogeneous, with the detection of at least five different HS species by antibodies generated via phage display (van Kuppevelt et al., 1998). The presence of an HS sequence in the kidney that promotes FR1c–FGF-4 complex formation, when combined with the knowledge that both FGF-4 and FR1c are expressed simultaneously in the developing kidney (Cancilla et al., 1999), supports the notion that HS has a regulatory role in FGF-4 signaling during kidney development. The fact that mice that lack the enzyme necessary for 2-*O*-sulfation of HS fail to develop functional kidneys may also implicate the HS in binding either FGFs or FRs (Bullock et al., 1998).

The lack of binding specificity in the case of FR1c recognition of FGF-2 or FR2c recognition of either FGF-2 or FGF-4 suggests several possibilities. In the case of FGF-2, it is likely that the minimum HS requirements necessary for receptor recognition are common components of HS biosynthesis. This is an intuitive result, as FGF-2 is one of the most widely expressed FGF family members and likely serves to signal in a wide variety of tissues and under a wide variety of physiological and pathological conditions (Baird, 1994; Szébenyi and Fallon, 1999). In this case, HS may serve as a facilitator of FGF-2 signaling rather than as a regulator. However, in the case of FGF-4, HS appears to be serving a regulatory role. It is clear that HS allows recognition of FGF-4 by FR1c in only very specific sites in the E18 stage embryo, whereas

FR2c recognizes FGF-4–HS on a much broader level. In each of these cases, it will be of interest to examine both FGF and receptor binding at earlier stages of development and in tissues where the FGFs and FRs are expressed and known to play a role, such as limb development in the case of FGF-4 (Martin, 1998). Further studies using other potential FGF receptors (including splice variants) as well as other FGF family members should identify additional specificity of these HS moieties in the regulation of FGF signaling.

Although it is widely accepted that HS is required for the formation of a high affinity FGF–FR signaling complex, there are few *in vivo* data regarding the ability of specific HS structures to regulate complex formation. The method used here provides a highly useful approach for mapping differences in HS structure and relating them directly to FGF activity. Although it is difficult to be certain that the binding of exogenous FGFs to the tissue sections is dependent only on the HS and is not influenced also by endogenous FRs, this seems unlikely as the FGFs bind to sites in the matrix where FRs are not expressed. In addition, we have shown that the putative FGF–HS complexes that are formed can be recognized by the exogenous FR probes. Furthermore, the predictions derived from the use of these FGF and FR probes are verified by activity studies using the BaF3 cells expressing FR1c or FR2c. Thus, it seems apparent that tissue-specific HS differentially regulates the binding of FGF-2 and FGF-4 in the developing mouse and also regulates the recognition of these FGFs by FR1c and FR2c in a tissue-specific manner. These results suggest a new paradigm where the formation of specific FGF–FR signaling complexes is regulated not only by the presence of HS, but also by site specific expression of distinct HS sequences necessary for complex formation.

## Materials and methods

### Preparation of FGF receptor–alkaline phosphatase fusion proteins

Soluble FGF receptor–alkaline phosphatase (FRAP) fusion proteins consist of the extracellular three Ig-like loop domains of the IIIc splice variants of either FR1 or FR2 fused to the NH<sub>2</sub> terminus of human placental-AP (FR1cAP and FR2cAP, respectively) (Ornitz et al., 1992). The FRAP cDNAs, provided by Dr. David Ornitz (Washington University, St. Louis, MO), were cloned into pcDNA3 and transfected into COS-7 cells using the calcium phosphate method and selected in 600 µg/ml Geneticin (G418 sulfate; GIBCO BRL).

FRAP protein was purified from conditioned medium on an anti-human placental AP-agarose column (Sigma-Aldrich) (Chang et al., 2000). FRAP immobilized on the column was washed with PBS containing 1 M NaCl to remove any endogenous HS that may have been bound to the receptor. The amount of active FRAP protein was quantified by measuring the AP activity of the samples using *p*-nitrophenyl phosphate (Ornitz et al., 1992). The activities of human placental AP standards (Sigma-Aldrich) of known concentrations were used to estimate FRAP concentration.

### *In vitro* heparan binding assays

Analysis of FRAP binding to FGF–heparin complexes was performed using HABs (Bio-Rad Laboratories). FRAP was incubated in tissue culture media (Hepes-buffered RPMI [HbRPMI] + 10% CS + L-gln) at a concentration of 100 nM with 10 µl HABs in either the absence or the presence of 30 nM human recombinant FGF-2, provided by B. Olwin (University of Colorado, Boulder, CO) or 30 nM human recombinant FGF-4 (R&D Systems) for 1 h at room temperature on a rotator. The HABs bearing FRAP were washed three times with either PBS or PBS containing 350 mM NaCl, resuspended in PBS, and loaded into 96-well plates with an equal volume of AP substrate solution and AP activity determined by absorbance at 405 nm. The amount of receptor bound was calculated as a percent of the total amount of receptor added to each treatment group.

### *In situ* HS binding assays

Frozen tissue sections were incubated for FGF detection as described (Friedl et al., 1997). Sections from E16 and E18 stage CD-1 mouse embryos (Charles River Laboratories) were cut at a thickness of 5 µm, air dried, and then fixed in 4% paraformaldehyde on ice. Two washes with cold 0.05% NaBH<sub>4</sub> followed by overnight treatment in PBS containing 0.1 M glycine at 4°C served to reduce autofluorescence of the tissues. Sections were blocked for 1 h at room temperature in TBS containing 10% fetal calf serum (Hyclone). Incubation of sections with FGF was for 1 h with 30 nM FGF-2 or 30 nM FGF-4. After three washes with TBS, bound FGF-2 was detected with 1:500 DE6 anti-FGF-2 antibody (a gift of DuPont), or bound FGF-4 was detected with 1:100 AF235 anti-FGF-4 antibody (R&D Systems) and Alexa-conjugated secondary antibodies (Molecular Probes).

Treatment of sections with 0.006 IU/ml heparin lyase I and heparin lyase III (referred to as heparitinase treatment) (Seikagaku America) for 2 h at 37°C, followed by addition of fresh enzyme for an additional 2 h completely removes endogenous HS. The unsaturated glucuronate remaining on the core protein is recognized by mAb3G10 (Seikagaku America), allowing examination of total HS distribution. Sections were incubated with 1:200 dilution of mAb3G10 in TBS containing 10% fetal calf serum for 1 h at room temperature, followed by 1:300 Cy3-conjugated donkey anti-mouse secondary antibody (Molecular Probes). Staining of sections without prior enzyme treatment shows no 3G10 staining (unpublished data).

For analysis of FRAP binding to FGF immobilized on endogenous HS, frozen sections were incubated with 30 nM native FGF-2 or FGF-4 for 1 h, then washed and incubated for an additional hour with 100 nM FR1cAP or FR2cAP in TBS containing 10% fetal calf serum. Sections were then treated with polyclonal rabbit anti-PLAP (Biomedica Corp.) for 30 min followed by Alexa 546-conjugated goat anti-rabbit antibody (Molecular Probes) for 30 min. Parallel sections were incubated with 1:10 fluorescein-conjugated PECAM-1 to identify capillaries. Parallel sections were incubated with 1:300 smooth muscle actin monoclonal antibody, a gift of Dr. Zsuzsa Fabry (University of Wisconsin–Madison, Madison, WI) to identify large blood vessels.

### Cell culture

MAECs were obtained from Dr. Robert Auerbach (University of Wisconsin–Madison, Madison, WI) and cultured in DME, 10% fetal calf serum, 4 mM L-glutamine, and 1% antibiotics (10,000 U penicillin/10 mg/ml streptomycin). Generation of Raji-S1 Burkitt's lymphoma cells has been described previously (Lebakken and Rapraeger, 1996). Raji cells are negative for proteoglycan expression and have been transfected with the cDNA for mouse syndecan-1. Raji-S1 cells were cultured in RPMI 1640, 10% fetal bovine serum, 4 mM glutamine, 1.5 mg/ml G418 sulfate, and 1% antibiotics. FR1c11 and FR2c2 cells, BaF3 lymphoid cells expressing FR1c and FR2c, respectively, were provided by Dr. David Ornitz. FR1c11 and FR2c2 cells were cultured in RPMI 1640, 10% fetal calf serum, 10% WEHI-3-conditioned medium, 4 mM L-glutamine, 1% antibiotics, and 0.0035% β-mercaptoethanol.

### Morphology assay

MAECs were plated in DME containing 10% fetal calf serum at a concentration of  $4 \times 10^3$  cells/well in 8-well chamber slides (LAB-TEK). After 24 h, cells were washed with serum-free DME, and incubated at 37°C in serum free DME with 10 nM FGF in the absence or presence of 10 nM porcine intestinal mucosa heparin (Sigma-Aldrich) for 72 h. Cells were then fixed in 1% glutaraldehyde and photographed.

### BaF3 proliferation assays

FR1c11 and FR2c2 cells were added to 96-well flat bottom plates (Fisher Scientific) at a concentration of  $10^5$  cells/ml in IL-3-deficient media. FGF (10 nM) was added and incubated at 37°C for 72 h in the presence or absence of 10 nM porcine intestinal mucosa heparin. After 72 h, CellTiter 96 AQueous One Solution reagent (Promega) was added to quantify relative cell numbers using the manufacturer's instructions.

For Raji-S1 cell-mediated survival/proliferation of the BaF3 cells,  $2.5 \times 10^6$  Raji-S1 cells/ml were allowed to adhere as a confluent monolayer to 96-well flat bottom plates for 2 h at 37°C in Hepes-buffered RPMI [HbRPMI], 0.1% BSA, 4 mM L-glutamine, and 1% antibiotics. The monolayer was fixed for 1 h in 0.5% glutaraldehyde, followed by three washes in PBS containing 0.2 M glycine, and incubated overnight in RPMI containing 10% CS, 4mM L-glutamine, and 1% antibiotics. FR1c11 and FR2c2 cells were added to the Raji-S1 monolayer the next day in IL-3-deficient media with or without FGF and treated as described above.

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