Specificity for Fibroblast Growth Factors Determined by Heparan Sulfate in a Binary Complex with the Receptor Kinase*

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A divalent cation-dependent association between heparin or heparan sulfate and the ectodomain of the FGF receptor kinase (FGFR) restricts FGF-independent trans-phosphorylation and supports the binding of activating FGF to self-associated FGFR. Here we show that in contrast to heparin, cellular heparan sulfate forms a binary complex with FGFR that discriminates between FGF-1 and FGF-2. FGFR type 4 (FGFR4) in liver parenchymal cells binds only FGF-1, whereas FGFR1 binds FGF-1 and FGF-2 equally. Cell-free complexes of heparin and recombinant FGFR4 bound FGF-1 and FGF-2 equally. However, in contrast to FGFR1, when recombinant FGFR4 was expressed back in epithelial cells by transfection, it failed to bind FGF-2 unless heparan sulfate was depressed by chlorate or heparinase treatment. Isolated heparan sulfate proteoglycan (HSPG) from liver cells in cell-free complexes with FGFR4 restored the specificity for FGF-1 and supported the binding of both FGF-1 and FGF-2 when complexed with FGFR1. In contrast, FGF-2 bound equally well to complexes of both FGFR1 and FGFR4 formed with endothelial cell-derived HSPG, but the endothelial HSPG was deficient for the binding of FGF-1 to both FGFR complexes. These data suggest that a heparan sulfate subunit is a cell type- and FGFR-specific determinant of the selectivity of the FGFR signaling complex for FGF. In a physiological context, the heparan sulfate subunit may limit the redundancy among the current 18 FGF polypeptides for the 4 known FGFR.

The FGF1 signal transduction system is ubiquitous and is a local mediator of developmental processes in the embryo and homeostasis in the adult (1). Through associations of both FGF and the FGFR ectodomain with heparan sulfate chains of pericellular matrix or transmembrane proteoglycans, the FGFR signaling complex senses perturbation and remodeling of the pericellular microenvironment (1). Heparan sulfate has been implicated in stability of FGF (2), access of FGF to the FGFR complex (3), oligomerization of FGF (4–6) and FGF-FGFR complexes (1, 4–8), and conformational repression and activation of FGFR oligomers (1, 9, 10). Heparin or heparan sulfate exhibits an FGF-independent, divalent cation-dependent high affinity interaction with immunoglobulin module II of self-associated oligomers of the FGFR ectodomain to form a complex that will bind FGF (8–10). Divalent cations and heparan sulfate cooperate to maintain the dependence of the FGFR complex on FGF (9), presumably by conformational restriction of the enzyme-substrate relationship between FGF, which otherwise are self-activating by trans-phosphorylation (1). A large number of genetically distinct FGF polypeptides have emerged, currently 18, for which there are only four FGFR (1). The high ratio of ligands to receptors, the co-expression of multiple FGFs within the same tissue or cells, and the apparent ability of the four FGF to bind multiple FGFs when heparin is used as the experimental mimic of heparan sulfate raise questions about the redundancy and functional relevance of the large number of FGF homologues. Here, we show that cellular heparan sulfate in a binary complex with the FGFR ectodomain is an FGFR isotype- and cell-specific determinant for selection of the FGF that interacts with the complex. In a physiological tissue context, this may restrict redundancy of the four FGFR for the large repertoire of FGF ligands.

EXPERIMENTAL PROCEDURES

Cell Culture, Preparation of Recombinant Receptors, and Radioreceptor Assays—The culture of primary rat hepatocytes collected by perfusion (11), HepG2 cells (12), and human endothelial cells has been described (13). Preparation and expression of recombinant FGF isoforms in the baculoviral-Sf9 system (10), preparation of FGFR-specific antibodies (14), and procedures for preparation and isolation, determination of specific binding, and covalent affinity cross-linking of radiolabeled FGF to infected Sf9 cells, mammalian cells, and immobilized purified recombinant FGF have also been previously described (9, 10, 15, 16). Recombinant FGFR4 was constructed as follows: P1 (5′-TTGG-GAAATTCCAGCTTGGTCCTCCT-3′) and P3 (5′-GGCCACGTATACGGAGCATCATC-3′) were used as sense primers beginning at 42 base pairs upstream of the translational initiation site and 15 base pairs upstream of the coding sequence for the transmembrane domain, respectively, using the polymerase chain reaction. P2 (5′-GGATGGAATTCACTTGC-3′) and P4 (5′-CACAGCCTGGACCTTGTCATG-3′) were used as antisense primers ending 124 base pairs downstream of the coding sequence for the transmembrane region and 18 base pairs downstream of the stop codon. Paired primers P1P2 and P3P4 were separately applied using HepG2 cell cDNA as template to amplify 1.3-kilobase extracellular and intracellular fragments, respectively. The P1P2 fragment was ligated into pBSK cloning vector at an 1.3-kilobase vector at an

* This work was supported by United States Public Health Service Grants DK40739 and DK35310 from the NIDDK, National Institutes of Health, and Grant CA59971 from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.‡ To whom correspondence should be addressed: Institute of Biosciences and Technology, Texas A&M University System Health Science Center, 2121 W. Holcombe Blvd., Houston, TX 77030-3303. Tel.: 713-677-7522; Fax: 713-677-7512; E-mail: wmkkeehe@ibt.tamu.edu.

† The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor kinase; HSPG, heparan sulfate proteoglycan; PBS, phosphate-buffered saline; Ch, chlorate; PAGE, polyacrylamide gel electrophoresis; Sf9, Spodoptera frugiperda; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonfonic acid.
Analyzing Specificity of Receptor Kinase by Heparan Sulfate

**RESULTS**

**Cell Type-dependent Specificity of FGFR4 for FGF-1—Cultured rat hepatocytes, which express exclusively FGFR4 (Fig. 1A), exhibit a mitogenic response to specifically FGF-1 (11, 18). Hepatocytes exhibit about 5000 FGFR sites/cell that specifically bind FGF-1 with a $K_d$ of about 60 pm (11, 18). The mitogenic response to or binding of FGF-2 in hepatocytes was undetectable (11, 18) (Fig. 1C). In contrast, hepatocyte-like human hepatoblastoma (HepG2) cells ectopically express isofoms of FGFR1, FGFR2, and FGFR3 in addition to FGFR4 (Fig. 1B), bind FGF-2, and exhibit 180,000 FGFR sites/cell that bind FGF-1 (12). However, an analysis with antiserum specific for FGFR1 and FGFR4 revealed that the FGFR1 fraction in HepG2 cells bound both FGF-1 and FGF-2, but the FGFR4 fraction bound only FGF-1 (Fig. 1D). This absolute specificity of FGFR4 for FGF-1 in liver parenchymal cells was similar to the selectivity of recombinant FGFR4 for FGF-1 reported in monkey kidney epithelial cells (19). However, it differed from FGFR4 in mouse fibroblasts (20), muscle cells (21), or leukemia cells (22), which bound both FGF-1 and FGF-2. Similar to the latter three mammalian cells, a recombinant complex of heparin and isolated recombinant FGFR4 derived from insect cells bound FGF-1 and FGF-2 equally (Fig. 2). Separate Scatchard analyses of the binding of FGF-1 and FGF-2 to binary complexes prepared with 1 $\mu$g of FGF, and the procedure was repeated. Separate Scatchard analyses of the binding of FGF-1 and FGF-2 to binary complexes prepared with 1 $\mu$g of FGF, and the procedure was repeated.

**Selectivity of Recombinant FGFR4 for FGF-1 Is Restored in Reconstituted Epithelial Cells and Abrogated by Depression of Heparan Sulfate**—To test the hypothesis that a cell type-dependent factor confers selectivity of FGFR4 for FGF-1, we reconstituted FGFR-deficient epithelial cells (A431 from a squamous cell carcinoma) with 1 $\times 10^5$ FGF1 or 5 $\times 10^4$ FGF4, and then examined the binding of FGF-1 and FGF-2 (Fig. 3). Scatchard analysis indicated that FGF-1 and FGF-2 bound to cells expressing FGFR1 with $K_i$ values of 104 $\pm$ 14 pm and 13 $\pm$ 9 pm, respectively, whereas cells expressing FGF4 bound only FGF-1 with a $K_i$ of 243 $\pm$ 24 pm. The binding of FGF-2 to FGF4 was below the limits of detection by both Scatchard analysis and covalent affinity cross-linking (Fig. 3A).

To determine whether cellular heparan sulfate played a role
in specificity of the binding of FGF in the reconstituted A431 cells, we applied sodium chloride, which inhibits sulfation of heparan sulfate, or heparinase, which modifies heparan sulfate chains. The treatment of the A431 cells expressing FGFR4 with sodium chloride reduced the binding of FGF to cellular heparan sulfate sites for FGF-1 and FGF-2 to 15 and 35%, respectively, of untreated controls. Surprisingly, the treatment resulted in the binding of FGF-2 to FGFR4 (Fig. 3B). This implicated heparan sulfate as the determinant of the cell type-dependent specificity of FGFR4 for FGF-1.

Parenchymal Cell Heparan Sulfate Complexed to Isolated FGFR4 Restores Specificity for FGF-1—We then tested whether the specificity of FGFR4 for FGF-1 was mediated by cellular heparan sulfate capable of binding to FGFR4 in the...
absence of cells and the pericellular matrix. Cell-derived heparan sulfate proteoglycan (HSPG) was released into the medium by treatment of cells with trypsin and partially purified by molecular filtration and anion exchange chromatography as described under “Experimental Procedures.” The activity of HSPG fractions was assessed by their ability to form a complex with the immobilized FGFR ectodomain after removal of unbound material and then by the ability of the resultant binary complex to bind radiolabeled FGF-1 or FGF-2 (8, 9, 16). The activity co-purified with macromolecules metabolically labeled with sodium [35S]sulfate, which ran ahead of the bulk of the protein on gel permeation chromatography (Fig. 4A). Activity and [35S]sulfate also co-purified during ion exchange chromatography slightly behind the main absorption peak at A280 (Fig. 4B). The partially purified fraction was radiolabeled with iodine as described under “Experimental Procedures” and analyzed by SDS-PAGE and autoradiography (Fig. 4C). On 7.5% gels, about 90% of radiolabeled material failed to penetrate the gel, whereas the rest of the material migrated in a band with mean apparent mass of about 75 (± 10) kDa (Fig. 4C, left panel). Treatment with heparinase resulted in a broad band of labeled material from 45 to 130 kDa. Treatment with Pronase destroyed 100% of the radiolabeled material. Analysis on 15% gels after treatment with heparinase (Fig. 4C, right panel) revealed that about 70% of the treated material migrated at apparent mass of 35 kDa that ran off the 7.5% gels. Separate experiments not shown here revealed that the FGF complementation activity survived treatment with both Pronase and chondroitinase A, B, C but was destroyed by treatment with heparinase or nitric acid. Inclusion of 8 M urea in the SDS-PAGE gels did not change the electrophoretic pattern of the indicated bands.

The iodinated partially purified heparan sulfate proteoglycan (125I-HSPG) was employed to further characterize direct binding to FGFR. Under FGF binding assay conditions, about 200 pg of the radiolabeled HSPG bound per ng of immobilized FGFR1. One hundred % of the 125I-HSPG that bound to immobilized FGFR1 was displaced by a 100-fold excess of heparin or treatment with 1 M NaCl. As reported previously, introduction of FGF into complexes of FGFR and heparin/heparan sulfate increased resistance of the bound heparin/heparan sulfate to displacement with external heparin, but not with 0.5 M NaCl (16). Displacement of 125I-HSPG bound to FGFR1 or FGFR4 was undetectable by either FGF-1 or FGF-2 at concentrations below 10 ng/ml. This was 2.5 times the standard amount of 125I-FGF used in binding assays and the highest concentration employed in the Scatchard analyses. Concentrations above 50 ng/ml of either FGF-1 or FGF-2 displaced 50% of the bound HSPG. Preliminary affinity chromatography experiments using immobilized FGFR1 columns (see under “Experimental Procedures”) indicated that a maximum of 20% of the 125I-HSPG can be retained on FGFR1 or FGFR4 at 0.15 M NaCl and that over 90% of the retained material, which elutes at 0.65 M NaCl, is in the high molecular weight fraction shown in Fig. 4A.

Additional experiments using the complementation assay with purified FGFR1 indicated that over 80% of the activity of the partially purified HSPG from HepG2 cells that bound to either FGFR1 or FGFR4 eluted from the immobilized FGFR between 0.25 and 0.65 M NaCl. This represented 7 and 5% of total material bound at 0.15 M NaCl to FGFR1 and FGFR4, respectively. About 65% of the FGFR1- or FGFR4-bound material that eluted between 0.25 and 0.65 M NaCl bound the other FGFR at 0.25 M NaCl after removal of the elution salt by dialysis. 75–85% of the recovered material bound back onto the homologous FGFR. Of the 0.25–0.65 eluate from FGFR1, 10 and 20% bound immobilized FGF-1 and FGF-2, respectively, at 0.25 M NaCl. About 10 and 46% of the HSPG from a similar eluate from FGFR4 bound FGF-1 and FGF-2, respectively. Of the total 125I-HSPG in the partially purified preparation from

![Image](326x311 to 536x729)

**FIG. 4.** Partial purification of cellular heparan sulfate proteoglycan fragments. A, separation by size. An extract of HepG2 cells (1.92 A280 units; 56,149 cpm of [35S]) prepared as described under “Experimental Procedures” was applied to the Biosil SEL-400 column and developed at a flow rate of 0.5 ml/min. B, anion exchange chromatography. Fractions under the solid bar from the molecular filtration chromatography in A were pooled and applied to a TSK-DEAE5PW column. The pool contained 5.9% of the A280 and 70% of the [35S] that was applied to the column. C, iodination and analysis of the partially purified HSPG fractions. A portion of the pooled fraction that was equivalent to 2.5 μg of heparin based on uronic acid content from A and B above was radiolabeled with 125I as described under “Experimental Procedures” and analyzed by SDS-PAGE on 7.5 and 15% gels followed by autoradiography. A 6.25-ng sample of the radiolabeled material (105 cpm) was subjected to treatment with 1.25 units of heparinase (Sigma, EC 4.2.2.7) or 25 μg of Pronase (Roche Molecular Biochemicals) at 37 °C for 1 h. N, no treatment; Hp, heparinase; Pr, Pronase.
HepG2 cells, 2 and 14%, respectively, bound to affinity columns of FGF-1 and FGF-2. Of the fraction that bound to immobilized FGF-1 that was eluted by 1.0 M NaCl, 18 and 14% bound to FGFR1 and FGFR4, respectively, at 0.25 M NaCl. Of the fraction that bound to immobilized FGF-2, 18 and 9% subsequently bound to FGFR1 and FGFR4, respectively. The results of purification of the HSPG fraction by combinatorial affinity chromatography using immobilized FGFR, FGF, and anti-thrombin will be reported elsewhere.

Scatchard analysis indicated that FGFR1 incubated with 0.2 μg/ml of the HSPG fraction supported FGF-1 and FGF-2 binding to isolated FGFR1 with a K_d of 60 pm (mean of two experiments) and 50 pm (mean of two experiments), respectively. The same fraction supported FGF-1 binding to isolated FGFR4 with a K_d of 140 pm (mean of two experiments). However, the binding of FGF-2 was insufficient to assign a K_d by Scatchard analysis. No radiolabeled FGF-2 could be detected by covalent affinity cross-linking, even when FGFR4 was incubated with more than 2 μg/ml of the HSPG fraction (Fig. 5, A and B). These results indicated that unlike heparin, cell-derived heparan sulfate restores the specificity of isolated FGFR4 for FGF-1 to that observed in the intact cell.

Finally, we isolated HSPG fraction by the same procedure from human umbilical vein-derived endothelial cells in which FGFR1 is a resident isoform. In the absence of exogenous heparin, the cell line binds and responds mitogenically to FGF-2, but only poorly to FGF-1 (13, 18). In contrast to the HSPG fraction from HepG2 cells, the fraction from the endothelial cells supported the binding of FGF-2 to both FGR1 and FGFR4 when tested in the FGFR complementation assay (Fig. 5, C and D). In contrast to heparin (Fig. 2) and liver cell HSPG (Fig. 5, A and B), the endothelial cell HSPG was deficient in support of the binding of FGF-1 to binary complexes of both FGFR1 and FGFR4.

DISCUSSION

The FGF signal transduction system is a ubiquitous and local mediator of embryonic development and homeostasis in adult tissues. It is a tripartite oligomeric complex composed of the transmembrane tyrosine kinase and heparan sulfate chains from pericellular matrix proteoglycan (1, 8–10). Pericellular matrix heparan sulfate plays multiple roles in assembly and activation of the FGFR complex. These roles include stability of FGF (2), access of FGF to the FGFR complex (9), and oligomerization of the FGFR complex through both the FGFR ectodomain and FGF (4–10). Heparin and heparan sulfate exhibit a divalent cation-dependent interaction with apparent K_d of about 10 nM to a specific sequence domain in Ig module II of the FGFR ectodomain (8–10). This is thought to restrict the trans-phosphorylation between self-associated FGFR kinases in the absence of FGF (8–10). In addition, the bound heparin/heparan sulfate is required and sufficient for the high affinity interaction of FGF with the binary complex in the absence of soluble heparin/heparan sulfate (8, 9, 23). These results support a model in which an activating FGF docks into inactive oligomeric complexes of FGFR and heparan sulfate chains from the pericellular matrix. In this report, we utilized this model of the stepwise assembly of the ternary complex of heparan sulfate-FGFR to determine whether the heparan sulfate subunit of the complex contributes to discrimination between FGFs.

Because there were conflicting reports concerning the discrimination of FGFR4 relative to FGFR1 for FGF-1 and FGF-2 among cell types (19–22), we employed FGFR4 and FGFR1 as experimental prototypes. FGFR4 is the sole isotype of the four known FGFR genes expressed in normal liver parenchymal cells and in that context, FGFR4 exhibits an absolute specificity for FGF-1 relative to FGF-2. In contrast, ectopically expressed FGFR1, which is co-expressed with FGFR4 in differentiated hepatoma cells, bound FGF-1 and FGF-2 equally. Yet reconstituted complexes of heparin and recombinant FGFR4, which were removed from cell membranes, pericellular matrix, and other cellular factors, bound both FGF-1 and FGF-2 in a manner similar to FGFR1. Expression of recombinant FGFR4 back into epithelial cells by transfection restored the specificity

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of FGFR4 for FGF-1. This suggested that the specificity was conferred by a cellular co-factor that was present in parenchymal cell lineages represented by the hepatocytes and HepG2 hepatoma cells, kidney epithelial cells (19), and the A431 squamous epithelial cells. The fact that the sulfation-inhibitor chlorate or treatment with heparinase abrogated the selectivity of FGFR4 for FGF-1 in intact cells suggested that cellular heparan sulfate was responsible.

Isolated and partially purified extracts containing cellular HSPG from the surface of the liver parenchymal tumor cell HepG2 substituted for heparin in formation of binary complexes with isolated recombinant FGFR1 and FGFR4 from insect cells. Similar to those formed with heparin, the binary complexes were then capable of binding FGF. Moreover, the isolated liver cell HSPG fraction restored the specificity of FGFR4 for FGF-1 to that observed in the liver parenchymal cells while continuing to support the binding of both FGF-1 and FGF-2 to FGFR1. This confirmed that the liver cells expressed a distinct HSPG that, when complexed with FGFR4, permits the high affinity docking of only FGF-1 into the complex. This explained the specificity of hepatocytes for FGFR-1.

Finally, we demonstrated that the discrimination for FGF conferred by HSPG when complexed to FGFR4 was cell-specific and, therefore, not a consequence of a general interference of FGFR4-bound HSPG with access of FGF-2 to the active site or general interference of FGF-2 with the binding of HSPG to FGFR4. In contrast to the liver cell HSPG, but similar to heparin, partially purified HSPG from endothelial cells supported the binding of FGF-2 to reconstituted complexes of both FGFR1 and FGFR4. Unlike liver cell HSPG and heparin, binary complexes of both FGFR1 and FGFR4 that formed with endothelial cell HSPG failed to efficiently bind FGF-1. This was consistent with the selective requirement for exogenous heparin for the binding and mitogenic response of endothelial cells to FGF-1 (13, 18). The results suggest that a deficiency in a HSPG subunit of the FGFR complex may limit the response of some endothelial cells to FGF-2.

Cell or tissue specificity of heparan sulfates in respect to affinity and differential effects on activity of FGF-1, FGF-2, and FGF-7 has been demonstrated (24–30). We conclude that FGFR- and cell type-specific pericellular matrix heparan sulfates complexed to the FGFR kinase ectodomain as an integral subunit may limit binding and activation of the FGFR signaling complex to one or a subset of the FGF ligands. In tissue and in a physiological context, this may limit the redundancy of the current 18 FGF polypeptide activators for the four FGF receptor kinases. Recently, we have demonstrated that only the fraction of commercial heparin and cellular heparan sulfate that binds anti-thrombin and inhibits Factor Xa activity can form a binary complex with the ectodomain of the FGFR kinase, and only the fraction enriched by recombimant FGFR affinity matrix exhibits anti-coagulant activities. Thus, the requirement for anticoagulant heparan sulfate and divalent cations (9) distinguishes the requirement for formation of the binary complex with all four FGF from the simpler requirements for the FGFR-independent binding to FGF. Currently, it is unclear whether FGFR1 and FGFR4 select heparan sulfate chains that discriminate between FGFs, or the selectivity for FGF is mediated by the same chain when bound to different FGFR. Although there appears to be a small fraction selected by both FGFR1 and FGFR4 that cannot bind the other, our preliminary results indicate that most of the liver cell-derived HSPG that binds either FGFR1 or FGFR4 can bind the other FGFR. On the one hand, the cross-reactivity may be due to multiple chains on the same HSPG fragment or multiple, but FGFR-specific, binding sites within a single chain. On the other hand, the composite complex formed by the same binding site within the same heparan sulfate chain in the two different FGFR may form the basis of the specificity for FGF. Clarification of the structural basis for the results requires purification of the specific HSPG underlying the activities through combinatorial affinity chromatography using FGFR, FGF and antithrombin columns, identification of the core protein sequence, and structural characterization of the heparan sulfate chains.

Acknowledgments—We thank Maki Kan, Kerstin McKeehan, and Thanh Tran for technical assistance.

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