Requirement for Anticoagulant Heparan Sulfate in the Fibroblast Growth Factor Receptor Complex*

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A divalent cation-dependent association between heparin or heparan sulfate and the ectodomain of the fibroblast growth factor (FGF) receptor kinase (FGFR) restricts FGF-independent trans-phosphorylation between self-associated FGFR and determines specificity for and mediates binding of activating FGF. Here we show that only the fraction of commercial heparin that binds to immobilized antithrombin formed an FGF-binding binary complex with the ectodomain of the FGFR kinase. Conversely, only the fraction of heparan sulfate that binds to immobilized heparan sulfate inhibited Factor Xa in the presence of antithrombin. Only the antithrombin-bound fraction of heparin competed with 3H-heparin bound to FGFR in absence of FGF, whereas both antithrombin-bound and unretained fractions competed with radiolabeled heparan bound independently to FGF-1 and FGF-2. The antithrombin-bound fraction of heparin was required to support the heparin-dependent stimulation of DNA synthesis of endothelial cells by FGF-1. The requirement for divalent cations and the antithrombin-binding motif distinguish the role of heparan sulfate as an integral subunit of the FGFR complex from the wider range of effects of heparan sulfate on FGF signaling through FGFR-independent interactions with FGF.

The FGF signal transduction system is ubiquitous and a local mediator of developmental processes in the embryo and homeostasis in the adult (1). Heparin or heparan sulfate interact independently with both activating FGF polypeptides, of which there are currently nineteen, and the ectodomain of the FGFR transmembrane kinases, which are encoded in four genes that give rise to multiple variants as a consequence of alternate splicing (1). Through the interactions, the FGF signal transduction system responds both negatively and positively to changes in the peri-cellular matrix. Heparan sulfate plays potentially multiple roles in FGF signaling in stability and proteolytic modification of FGF (2), in control of access of FGF to the FGFR kinase complex (3, 4), oligomerization of FGF (5–7) and FGFR complexes (1, 5–7), and in conformational activation of oligomers of FGFR complexes (1, 9). Heparin-derived oligosaccharides ranging from simple unsulfated disaccharide to trisaccharide units to sulfated six to ten units have been co-crystallized with FGF (7, 8, 10) which enhance oligomerization and affect FGF activity at the cellular level. Others have argued that FGF-2 associates more specifically with a pentasaccharide containing glucosamine-N-sulfate and a single iduronic acid-2-O-sulfate (11, 12). The 6-O-sulfate of glucosamine-N-sulfate residues may contribute to the interaction with other FGFs (7, 14). A longer oligosaccharide that contains glucosamine-N-sulfate (6-O-sulfate) is more active in enhancing the interaction of FGF with FGFR and the activities elicited by FGF in various bioassays (12–17). The additional length has been proposed to reflect the requirement for spanning FGF dimers that bind an FGFR monomer or for spanning two FGFs that bind adjacent FGFR kinases (5–8). The length and 6-O-sulfate requirement may also reflect requirement for a bivalent interaction with FGF and FGFR to form a ternary unit (1, 9, 18). The structural restrictions within heparan sulfate required for formation of an FGF-binding binary complex with the FGFR kinase or the ternary complex with both FGF and FGFR are less clear than the independent interaction with FGF. Characterization of the structural requirements in heparan sulfate for association with the FGFR kinase has been hampered in vivo by the interference with cellular heparan sulfates and in vitro by structural instability of isolated FGFR and the variability in the dependence on heparin/heparan sulfate for FGF binding (9). Recently we showed that divalent cations stabilize the FGFR ectodomain, squelch the heparin/heparan sulfate-independent FGF binding and mediate the high affinity interaction of heparin/heparan sulfate to FGFR (9). This interaction restricts activating trans-phosphorylation between self-associated FGFR in absence of FGF (9) but is required for and can mediate selectivity of binding of the activating FGF (19). Using these improvements, we report here that, in contrast to the interaction of heparin/heparan sulfate with FGF, the functional complex with the FGFR kinase ectodomain requires all or a part of the structural motif that binds to antithrombin.

EXPERIMENTAL PROCEDURES

Binding of 125I-FGF to Heparin- or Heparan Sulfate Proteoglycan (HSPG)/FGF Receptor Complexes—Purified FGFR1β-GST was immobilized on GSH-agarose beads (Glutathione-Sepharose 4B, Amersham Pharmacia Biotech, Uppsala, Sweden) and incubated with heparin (No. H-3393, molecular weight 6,000–20,000, 195.2 USP units/mg from porcine intestinal mucosa) or rat liver HSPG in PBS containing 1% Triton X-100 and 10 mM MgCl2 for 1 h at room temperature (9, 19). After washing extensively, 250 µl of 125I-FGF-1 or 125I-FGF-2 (4 ng/ml at specific activity of 3.2 and 1.6 x 106 cpm/µg, respectively) was added for 1 h at room temperature, the beads were washed with PBS three times, and the radioactivity was determined by γ-counter. The complex of 125I-FGF and FGFR was then chemically cross-linked by disuccinimidyl carbamidate and detected by autoradiography after SDS-polyacrylamide gel electro-
phoresis. Recombinant human FGF-2 was from Upstate Biotechnology, Inc. (Lake Placid, NY). FGF-1 was purified from bovine brain and the FGFRC1β ectodomain fused to glutathione S-transferase (FGFRC1β-GST) was expressed in S99 cells as described (9). Inhibited FGF-1 and FGF-2 were prepared as described (20). Unless otherwise indicated, data points in text illustrations were the mean of duplicates both for radioabeled FGF and heparin binding. The experiments were representative of at least three reproductions using independent preparations of fractionated heparin or HSPG. At least two experiments were performed with different preparations of radioabeled FGF.

**Antithrombin (AT) and FGF Affinity Chromatography of Heparin and HSPG**—Heparin (1 µg) and 0.1 µg of 125I-heparin (0.41 mCi/mg; molecular weight 6,000–20,000, 142.5 units/mg from porcine intestinal mucosa from NEN Life Science Products, Boston, MA) was mixed with antithrombin III-agarose beads (Sigma) (0.2 ml) in 1 ml of PBS containing 1% Triton X-100 and 10 mM MgCl₂ for 2 h at room temperature under constant shaking. The beads were then washed with the buffer extensively, and the bound heparin was eluted with 1 M NaCl in the buffer. The eluted fraction was dialyzed against the PBS buffer for assay. The bound heparin was about 14% of total heparin applied. Rat liver HSPG (10 µg) was prepared and partially purified as described below and then similarly fractionated by AT affinity chromatography. About 8% of the partially purified HSPG was retained on the column. 

**1H-Heparin (0.1 µg) was added to 5 µg unlabeled heparin in 1 ml of 1% Triton X-100 containing 10 mM MgCl₂ in PBS and applied to an FGFRC1β-GST affinity column with packed bead volume of 0.4 ml (2–4 µg FGF) prepared as described (19). The bound heparin was eluted with 1 M NaCl in PBS. Unretained material was desalted and repeatedly run on the column until bound heparin was negligible. FGFR extracted about 3% of the heparin applied. The FGFR-bound heparin was desalted and used for subsequent analysis.**

**Competition of the AT-binding Fraction of Heparin or HSPG with 3H-Heparin Bound to FGFR or FGF**—Competitive binding assays were performed by incubation of immobilized FGFRC1β or FGF with 0.5 ml of 3H-heparin (0.1 µCi/ml) in PBS containing 1% Triton X-100 and 10 mM MgCl₂ in the presence or absence of different concentrations of unlabeled heparin or fractions from heparin for 1 h at room temperature. After washing the beads with buffer three times, the bound radioactivity was extracted by 0.5 ml of 1.5 NaCl in PBS and counted by liquid scintillation. FGFRC1β-GST was immobilized on GST-Sepharose beads, and FGF was immobilized on copper-chelating beads (Chelating Sepharose Fast Flow, Amersham Pharmacia Biotech) as described (19).

**Purification of HSPGs from Rat Liver**—Male rat livers (F-344) were perfused with 100 ml of 10 µg/ml trypsin and 0.02% EDTA in PBS for 10 min at room temperature after similar treatment without trypsin. The perfusate was clarified by centrifugation, dialyzed against water, and freeze-dried. The solid was then reconstituted with 1 ml of PBS and fractionated by gel-permeation (Bio-sil SEC-400, Bio-Rad, Richmond, CA) and ion exchange (Bio Gel TSK-DEAE-5-PW BIO-RAD, Richmond, CA) and high performance liquid chromatography as described (19). The activity of fractions was determined in the FGFR assembly assay, which measures both ability to form an FGF-independent binary complex with immobilized FGFR1β and the subsequent binding of radio labeled FGF-1 and FGF-2 to the complex. Active fractions were pooled, dialyzed against water, lyophilized, and reconstituted in PBS. The carbohydrate concentration was determined by the carboxymethyl (21).

**Inhibition of Factor Xa Activity**—Fractions indicated in the text and 1 µg/ml antithrombin (Calbiochem-Novabiochem International, San Diego, CA) were added to assays containing 1 ml of 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 10 mM calcium chloride, 1.0 µl of Factor Xa (10 µg/ml, New England Biolabs, Beverly, MA) and 25 µl of chromogen X (11.65 mg/ml, Roche Molecular Biochemicals, Mannheim, Germany). Incubation was carried out for 2 h at room temperature, and the absorption at 405 nm was measured.

**RESULTS**

The AT-binding Fraction of Heparin Is Required for Formation of FGF-binding Complexes of Heparin and FGFRC1β—About 1% of size- and charge-enriched liver cell heparan sulfate proteoglycan with FGF complementation activity in cell growth binding assays binds to immobilized FGFRC1 or FGFRC4 under optimized conditions (9, 19). A similar analysis revealed that about 3% of a commercial heparin preparation bound to immobilized FGFRC1 under the same conditions. Thus about 97% of heparin added into binding assays in soluble form may be incapable of participating in formation of a ternary FGFR complex. However, 5 and 15% of the heparin that is unretained by immobilized FGFRC1 still binds FGF-1 or FGF-2, respectively, at 0.5 M salt (not shown). An even higher proportion binds at the 0.15 M salt employed in binding assays (50–70% to FGF-2 and about 30% to FGF-1). The binding of 125I-FGF-1 or 125I-FGF-2 to purified and immobilized FGFRC1β-GST was employed to determine the structural requirements within the minority fraction of heparin and heparan sulfate that formed a divalent cation-dependent binary complex with FGFRC (9, 18, 19). Because the portion of commercial heparin that exhibits anticoagulant activity represents a distinct structural subset, porcine intestinal heparin was fractionated by AT-affinity chro-
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matography. Activity of the bound and unbound fractions was analyzed for ability to bind to immobilized FGFR1β and the support of the binding of FGF to the immobilized binary complex (Fig. 1, A and B). Surprisingly, only the fraction of heparin that was retained by the AT column exhibited activity. Specific activity was increased by 8–10-fold. Activity of the fraction of heparin that failed to bind to the AT column was below detection limits, even when the immobilized FGFR1β was incubated with up to 0.4 μg per ml of heparin. Similar results were observed in separate experiments using immobilized FGF2βIIIb-GST and FGF-1 and FGF-7 as radiolabel, and for FGFR4 with both FGF-1 and FGF-2 (results not shown).

To determine whether the immobilized FGFR selected the anticoagulant fraction of heparin, the heparin that was captured by immobilized FGFR was recovered and assayed for ability to inhibit Factor Xa in the presence of antithrombin. Only the fraction of heparin (B) extracted by the immobilized FGFR exhibited anticoagulant activity as assessed by inhibition of Factor Xa activity in the presence of AT (Fig. 1C). Separate experiments confirmed that AT-bound heparin was 5 to 7 times more potent than crude heparin and that 1 and 10 ng/ml AT-bound heparin completely inhibited Factor Xa activity under the conditions indicated.

The AT-bound Fraction Selectively Competes with 3H-heparin Bound to FGFR—To confirm that the selective activity of the AT-bound fraction of heparin for formation of an FGF-binding complex with FGFR1β reflected the FGF-independent interaction with FGFR1β, the AT-bound and unretained fractions were tested for ability to compete with receptor-bound radiolabeled heparin (Fig. 2A). Similar to the results from the FGF binding assays, only the AT-bound fraction of heparin competed with FGFR-bound heparin.

To determine the competition of the two heparin fractions to 3H-heparin bound to FGF in absence of AT, FGF-1 and FGF-2 were immobilized on copper-chelating Sepharose beads, which then bound 3H-heparin. Fig. 2B shows that, although the AT-bound fraction of heparin was more efficient, both AT-bound and unbound fractions competed with heparin bound to FGF.

Only the AT-binding Fraction of Rat Liver HSPG Forms an FGF-Binding Complex with FGFR1β—Native HSPG from rat liver was collected by perfusion, partially purified by gel filtration and ion exchange chromatography, and then fractionated by AT affinity chromatography. Similar to heparin, only the fraction that was retained on the AT column exhibited the ability to form an HSPG-FGFR1β complex that bound either FGF-1 or FGF-2 (Fig. 3).

The AT-bound Heparin Selectively Supports the Mitogenic Activity of FGF-1 for Human Umbilical Vein Endothelial Cells (HUVEC)—The stimulation of DNA synthesis of HUVEC by FGF-1 exhibits a stringent requirement for added heparin, whereas the stimulation by FGF-2 is relatively independent (22, 23). This appears to be because of a deficiency of a cellular HSPG that will form a binary complex with FGFR1 that is competent to bind FGF-1 (19). The AT-bound fraction of hepa-
rin enhanced FGF-1-induced DNA synthesis, whereas the unretained fraction exhibited no activity (Fig. 4). In separate experiments not shown here, we have demonstrated that soluble antithrombin inhibited both basal and FGF-1- and FGF-2-stimulated DNA synthesis of the endothelial cells in a dose-dependent fashion. Moreover, antithrombin at 10–20 μg/ml inhibited 125I-FGF binding to the cells by 60%. These observations are consistent with the possibility that antithrombin competes with endogenous heparan sulfate that forms an obligatory binary complex with FGFR, although alternative activities of antithrombin through other mechanisms cannot be eliminated.

DISCUSSION

Oligosaccharides with the structural motif associated with the anticoagulant activities of heparin or heparan sulfate, which requires 3-O-sulfation, appear to be unnecessary for the FGFR-independent interaction with FGF-1 and FGF-2 (7, 8, 10–17). In this report we examined the direct association of heparin and heparan sulfate with the purified recombinant ectodomain of the FGFR kinase. This enabled study of the structural requirements required to form a binary complex that is competent to bind FGF in the absence of interfering cellular heparan sulfates or soluble heparin, or heparan sulfate that binds FGF, but is incapable of interaction with FGFR. The results revealed that only the fraction of heparin or cell-derived heparan sulfate that was competent to bind AT and to inhibit factor Xa in presence of AT was capable of forming a competent binary complex with FGFR. The results suggested that the FGFR kinase ectodomain specifically selects, from unfraccionated heparin and heparan sulfate, the fraction that exhibits anticoagulant activity.

The structural motif within heparin that is required for AT binding and anticoagulant activity is a penta- or hexasaccharide sequence, which can be up to 30% of unfraccionated heparin, but is less than 10% of cellular heparan sulfates (24). A glucosamine-N-acetyl or N-sulfate-6-O-sulfate and a glucosamine-N-sulfate-3-O-sulfate (± 6-O-sulfate), with a residue in between, cooperate with an adjacent disaccharide comprised of iduronic acid-2-O-sulfate and glucosamine-N-sulfate-6-sulfate in AT binding (24). It is likely that all or a part of this structure within heparin or heparan sulfate participate in the specific divalent cation-dependent interaction with the FGFR ectodomain. However, it is noteworthy that less than 50% of AT-bound heparin will subsequently bind to FGFR1. This suggests a structural requirement in addition to the AT-binding motif for formation of the binary FGFR complex.

Recently, it has become clear that tissue-specific and hormonally regulated isozymes of glucosaminyl-3-O-sulfotransferases (3-OST) are the final and rate-limiting step in heparan sulfate synthesis which generates 3-O-sulfate sites in positions dictated by the oligosaccharide sequence in precursors (24–26). Activity of these enzymes may be intimately involved in both the negative and positive regulation of FGF signaling through modification of the composition of heparan sulfate chains of the proteoglycan subunits of the oligomeric FGFR complex. The requirement for divalent cations and the AT-binding motif within heparin or heparan sulfate for formation of competent FGFR glycosaminoglycan-kine complexes distinguish the FGFR complex from other indirect actions of heparin or heparan sulfate. This property should aid in characterization of the responsible proteoglycan based on the properties of its glycosaminoglycan chains.

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