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Ligand Binding Properties of Binary Complexes of Heparin and Immunoglobulin-like Modules of FGF Receptor 2

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Epithelial cells, which express FGFR2IIIb, bind and respond to FGF-1, FGF-7 and FGF-10, but not FGF-2. Stromal cells, which bind and respond to FGF-1 and FGF-2, but not FGF-7 and FGF-10, express FGFR2IIIc or FGFR1IIIc. Here we show that when both isolated FGFR2βIIIb and FGFR2βIIIc or their common Ig module II are allowed to affinity select heparin from a mixture, the resultant binary complexes bound FGF-1, FGF-2, and FGF-7 with nearly equal affinity. In addition, FGF-2 and FGF-7 bound to both heparin-Ig module IIIb and IIIc complexes, but FGF-1 bound to neither Ig module III. The results show that in isolation both Ig modules II and III of FGFR2 can interact with heparin and that each exhibits a binding site for FGF. We suggest that the specificity of FGFR2IIIb and FGFR2IIIc is dependent on the cell membrane environment and heparin/heparan sulfate. Ig modules II and III cooperate both within monomers and across dimers with cellular heparan sulfates to confer cell type-dependent specificity of the FGFR complex for FGF. **© 2000 Academic Press**

Key Words: **extracellular matrix; heparan sulfate proteoglycans; tyrosine kinases; prostate cancer; stromal–epithelial interactions; cell communication.**

Communication between stromal and epithelial compartments of parenchymal organs is critical to maintenance of homeostasis and function, and miscommunication underlies diverse pathologies including cancer. Among the 22 FGF polypeptides studied to date, FGF-7 exhibits a restricted expression in stromal cell types and specificity for the resident splice variant FGFR2IIIb in epithelial cells (1). Conversely, FGFR2IIIb exhibits a restricted specificity when expressed in epithelial cells. In addition to FGF-7, it recognizes only FGF-1, which interacts with multiple FGFR isotypes, and FGF-10, a stromal cell-derived homologue of FGF-7 (2). The lack of binding of FGF-7 and FGF-10 to FGFR1 and FGFR2IIIc in stromal cells in an autocrine mode contributes to the directional paracrine signaling from stroma to epithelium via FGF-7/FGF-10 and FGFR2IIIb (1). A wealth of biochemical data has been generated to show how mutually exclusively spliced alternate exons IIIb and IIIc of FGFR2, which code for the second half of Ig module III, contribute to the respective mutually exclusive specificity for FGF-7 and FGF-2 in diverse test systems. Although FGFR1β, which is comprised of Ig modules II and III, binds FGF-1 and FGF-2, but not FGF-7, a chimeric construct of FGFR1 containing exon IIIb of FGFR2 rejects FGF-2 and binds FGF-1 and FGF-7 (3, 4). This suggested that the FGFR2IIIb exon is the major determinant of specificity for FGF-2 and FGF-7. Upstream domains in both FGFR1 and FGFR2 support the binding of both FGF-2 and FGF-7. Tryptic fragments and mutant constructs of FGFR1 and FGFR2, which are devoid of sequences in Ig module IIIc downstream of lys-189, bind FGF-1, FGF-2 and FGF-7 (4). This confirmed the ability of domains in both FGFR1 and FGFR2 to bind all three ligands in absence of exon III sequences and suggested a restrictive role of variant sequences in exon IIIb and IIIc on FGF-2 and FGF-7 binding, respectively (4). The binding of FGF-7 to chimeric constructs of Ig module IIIb of FGFR2 at

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the C-terminus of two Ig modules (C_H) from the immunoglobulin heavy chain has been demonstrated in absence of Ig module II (5). Subsequent experiments from our laboratory confirmed that isolated Ig module II from FGFR1, in absence of restrictive sequences at the C-terminus, is capable of binding FGF-1 and FGF-2 and to some extent, FGF-7 (6, 7). Taken together, these results suggested that the structural Ig modules II and III might exhibit independent binding sites.

Recently we reported that only about 1% of size- and charge-enriched cellular heparan sulfate and 3% of commercial heparin (molecular weight 6000–20000) forms a binary complex with isolated FGFR that is competent to bind FGF (8, 9). In contrast to the independent interaction with FGF-1 and FGF-2, formation of the binary FGFR–sugar complex requires heparin or heparan sulfate that exhibits antithrombin-binding and anticoagulant activity (8, 9). At physiological salt concentrations, FGF-1 and FGF-2 can bind up to 30 and 70% of crude heparin or cellular heparan sulfate, respectively, relative to the 1 to 3% that binds to FGFR (8, 9). These observations suggest that the 97 to 99% of crude heparin or heparan sulfate, both free and complexed with FGF, that cannot interact with FGFR may interfere with the quantitative analysis of the assembly of the ternary complex between FGFR, heparin and FGF.

In this report, we employed pre-formed heparin–FGFR complexes to re-assess quantitatively the contribution of variant isoforms of FGFR2 and its isolated Ig modules to binding of FGF-1, FGF-2 and FGF-7 in absence of soluble heparin. The results revealed that (i) both Ig modules II and III appear to interact with heparin/heparan sulfate; (ii) isolated Ig modules II and III exhibit independent binding sites that differ in affinity for FGF; and (iii) binary complexes of neither FGFR2βIIIb or IIIc isoforms nor their derived Ig modules exhibit strictly the same FGF-binding profile observed in the presence of soluble heparin or the strict specificity for FGF observed in specific cell types.

MATERIALS AND METHODS

Construction and expression of FGFR2 structural modules. A schematic of FGFR2 constructs is shown in Fig. 1. cDNA coding for the extracellular domain of the rat FGFR2βIIIb isoform (4, 10) was used as a template in the polymerase chain reaction (PCR) using the oligonucleotide primers listed below. Restriction sites are underlined and nucleotides not in the coding sequences for FGFR or GST are in lower case. The GST portion of each construct was prepared in the PCR using the GSTB and GSTE primers and the pGEX-2T vector (Amersham Pharmacia Biotech, Piscataway, NJ) as a template. The amplified fragments were purified by 1 to 2% agarose gel electrophoreses and then digested with *Bam*HI and *Eco*RI. The FGFR2 portion of R2βIIIb-GST and R2βIIIc-GST was prepared by treating R2βIIIb and R2βIIIc cDNA with *Pst*I and *Bam*HI, respectively. For the R2L2-GST construct, 5PST-3R2D and 5R2E-3R2 fragments were prepared in the PCR. 5PST-3R2D was treated with *Pst*I and *Kpn*I, and 5R2E-3R2 was treated with *Kpn*I and *Bam*HI. For the R2L22-GST construct, fragments 5PST-3R2L22 and 5R2E-3R2 were prepared. 5PST-3R2L22 was then treated with *Pst*I and *Kpn*I, and 5R2E-3R2 was treated with *Kpn*I and *Bam*HI. For R2L3IIIb-GST, the 5PST-3R2S fragment was prepared by PCR and treated with *Pst*I and *Kpn*I. The 5R2C-GSTE fragment was prepared using R2βIIIb-GST as a template and digested with *Kpn*I and *Eco*RI. For R2L3IIIc-GST, the 5R2C-GSTE fragment was prepared using R2βIIIc-GST as a template. The PCR product was digested by the indicated restriction endonucleases and cloned into the multiple cloning site sequence of the insect cell expression vector pVL1392 (Invitrogen Co., San Diego, CA). 5R2C, 5′ CTT GAT GTT GTT ggt acc GAG CGA TCA CCA CAC CGG 3′; 5R2E, 5′ CTC ACT GTC CTG ggt acc CCC AAA CAG CAA GCA 3′; 3R2D, 5′ TGG TGA TCG CTC ggt acc AAC AAC ATC AAG GTG GTA 3′; 3R2S, 5′ ACT GAA GGA GGG ggt acc CCG GGC CAG GGA CAA 3′; 5PST, 5′ AAA ctg cag AGG ACC AGG GAT TGG 3′; 3R2L22, 5′ CAC CGT GGA GGC ggt acc ATT TGC AGG CAG 3′; GSTB, 5′ TTC ATG gaT CCT ATA CTA GGT GGT TAT 3′; GSTE, 5′ ACG ATG AAT TCC CGG tcA TCC ACG 3′.

Expression and purification of recombinant FGF-7. The 17 kDa FGF-7β (^{54}Ser-FGF-7) isoform was prepared in bacteria by expression of the coding sequence for bacterial glutathione *S*-transferase (GST) fused in frame with that of FGF-7 beginning at Ala-30 in vector pGEX-2T (Amersham Pharmacia Biotech) as described (11). A 23 kDa isoform with the amino-terminal sequence gly-ser-ala-cys-asn, which we call FGF-7α, was prepared by the same procedure from a thrombin cut site placed between GST and the FGF-7 amino terminus. The purity of product was judged to be 95% by SDS–polyacrylamide gel electrophoresis and amino-terminal sequence.

^{125}I-FGF binding to immobilized complexes of FGFR and heparin. Recombinant baculovirus encoding FGFR2-GST constructs were prepared, viral stocks were standardized and maintained, and viruses transfected into Sf9 insect cells as described (8, 9). Binary complexes of recombinant FGFR2-GST constructs were prepared, extracted and immobilized on GSH-beads and the analysis of binding and covalent affinity crosslinking of ^{125}I-FGF was done as described previously (8).

Scatchard analysis. Dissociation constants (K_ds) were determined by Scatchard analysis (1, 8). Immobilized binary FGFR complexes were prepared as described above with 1 μg/ml heparin and introduced into binding assays containing ^{125}I-FGF. ^{125}I-FGF was added at concentrations from 1 to 100 ng/ml. Separate competition experiments using unlabeled FGF indicated that non-specific binding at 10 ng/ml was about 10% for all constructs complexed with heparin. K_d values ($\pm$ standard error) were estimated by linear least-square analysis.

RESULTS AND DISCUSSION

Differences in the binding of FGF in presence of soluble heparin or pre-formed binary complexes of heparin. cDNAs coding for the indicated isoforms and Ig modules of FGFR2β (Fig. 1) were fused to GST, expressed on the surface of baculoviral-infected insect cells and then extracted and immobilized on GSH-beads. The binding of radiolabeled FGF-1, FGF-2 and FGF-7 to the immobilized products was compared in the absence (N) or presence of heparin. Heparin was introduced in the assays in soluble form (S) or in bound form (B) in binary complexes prepared by preincubation and removal of unbound soluble heparin prior to introduction of labeled FGF (Fig. 2). At concentrations of heparin greater than 10 ng/ml, the binding of FGF-1 and FGF-2 to binary complexes of FGFR1βIIIc (here-

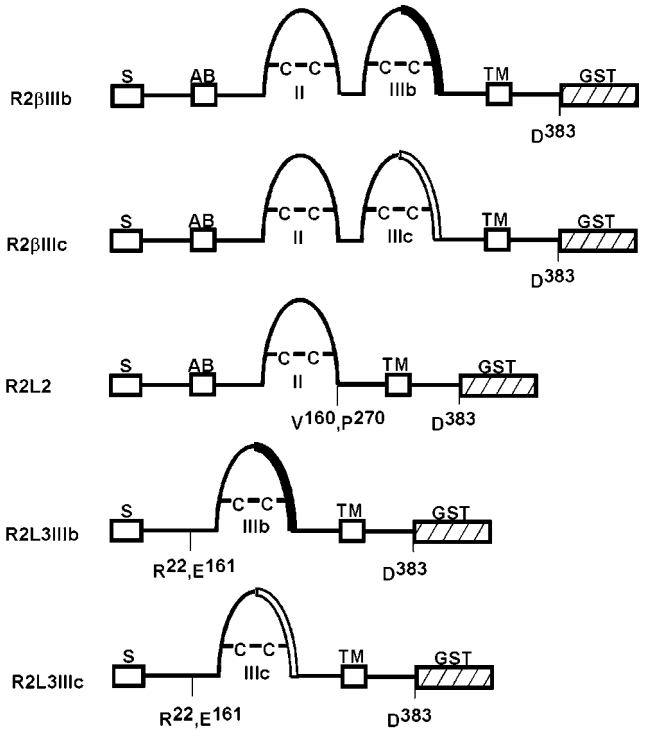

FIG. 1. Recombinant FGFR2 constructions. Ig modules II and III are indicated with the interloop disulfide. Exons IIIb and IIIc are indicated by the thick black and white lines, respectively. The numbering of the amino acids is based on the predicted product beginning at the initiator methionine of the rat full-length FGFR2IIIb cDNA isoform containing two Ig modules (FGFR2βIIIb) (4). The secretory signal sequence (S), the acidic box (AB), and the transmembrane domain (TM) are indicated. The GST sequence that begins at D383 of FGFR2β is indicated by the hatched rectangle.

after referred to as FGFR1β) or FGFR2β and heparin was markedly enhanced 3- to 7-fold over binding in the presence of soluble heparin (Fig. 2). Although the binding of FGF-7 to FGFR1β was undetectable, the binding of FGF-2 to FGFR2βIIIb and FGF-7 to FGFR2βIIIc in binary complexes with heparin was particularly notable (Fig. 2). This promiscuity of the two isoforms for FGF-2 and FGF-7 is not observed in native epithelial or stromal cells.

Scatchard analysis of binding to the FGFRβ isoforms confirmed that the positive binding results reflected saturable, high affinity binding with K_ds in the pM range (Table 1). Covalent affinity crosslinking and autoradiographic analysis of the FGF-labeled complexes confirmed that the binding was to high affinity sites on the FGFR kinase ectodomain, not simply to the bound heparin chain which does not crosslink to FGF (Fig. 2). These results show that, in contrast to FGFR1β, which stringently rejects FGF-7 under all conditions, absolute specificity for either FGF-2 or FGF-7 is not encoded in the structure of either isoform of FGFR2. Apparent specificity depends on the nature and order of addition of the heparin/heparan sulfate co-factor in binding assays *in vitro*.

Binding of FGF-1, FGF-2 and FGF-7 to binary complexes of heparin and isolated Ig module II of FGFR2. In previous studies, we showed that fragments of FGFR1β and FGFR2β containing an intact module II, but truncated upstream of the alternately spliced COOH-terminus of Ig module III, bound FGF-1, FGF-2 and FGF-7 (4). However, sequences between the COOH-terminus of module II and the truncations (4) or foreign fusion sequences (5) negatively impacted the binding of FGFs in order of sensitivity FGF-7 $>$ FGF-2 $>$ FGF-1. When all sequences of COOH-terminal to Ig module II of FGFR1 were removed, the module bound FGF-1, FGF-2 and FGF-7 in respective decreasing order of efficacy (4, 7). Detection of FGF-7 binding required a 10 times higher concentration of radiolabeled FGF-7 with 10 times longer exposure of autoradiographs. In these experiments, the COOH-terminal boundary of the Ig module II was arbitrarily defined by the exon-exon junction coding for the COOH-terminus of module II and the beginning of the inter-Ig module domain (Fig. 1). This has been recently validated by crystal structures (12, 13). Here we examined the binding of the three radiolabeled FGFs to a construct comprised of the amino terminus including the secretory signal of FGFR2, Ig module II, the extracellular juxtamembrane and transmembrane domain of FGFR2 fused to GST (Fig. 1). Binding was examined in the presence of soluble heparin (S) at 1 μg/ml or to complexes formed by affinity selection of heparin from the soluble mixture at 1 μg/ml. The construct (R2L2) bound FGF-1 in the presence of soluble heparin, but with maximal FGF-1 binding to pre-formed complex generated with the same amount of heparin (Fig. 3). In contrast, the binding of FGF-2 and FGF-7 was nearly undetectable in the presence of soluble heparin (Fig. 3), but exhibited an apparent K_d of 86 $\pm$ 18 and 70 $\pm$ 20 pM, respectively, to the heparin-module II complex (Table 1). Covalent affinity crosslinking analysis verified that at least a component of the binding was the high affinity interaction of radiolabeled FGF with the recombinant constructs (Fig. 3). The binding of FGF-7 to Ig module II from FGFR1 was undetectable under the same conditions although, as mentioned above, some FGF-7-labeled complex can be detected by prolonged exposure of autoradiographs (4, 7). These results show that, similar to Ig module II from FGFR1, Ig module II from FGFR2 in complex with heparin can bind all three FGFs if soluble heparin is removed after affinity selection by pre-incubation with the heparin mixture. Module II from FGFR2 exhibits a much higher affinity for FGF-7 than module II from FGFR1. This is consistent with the report of Zimmer *et al.* (3) that showed that FGFR2IIIb exhibits higher affinity

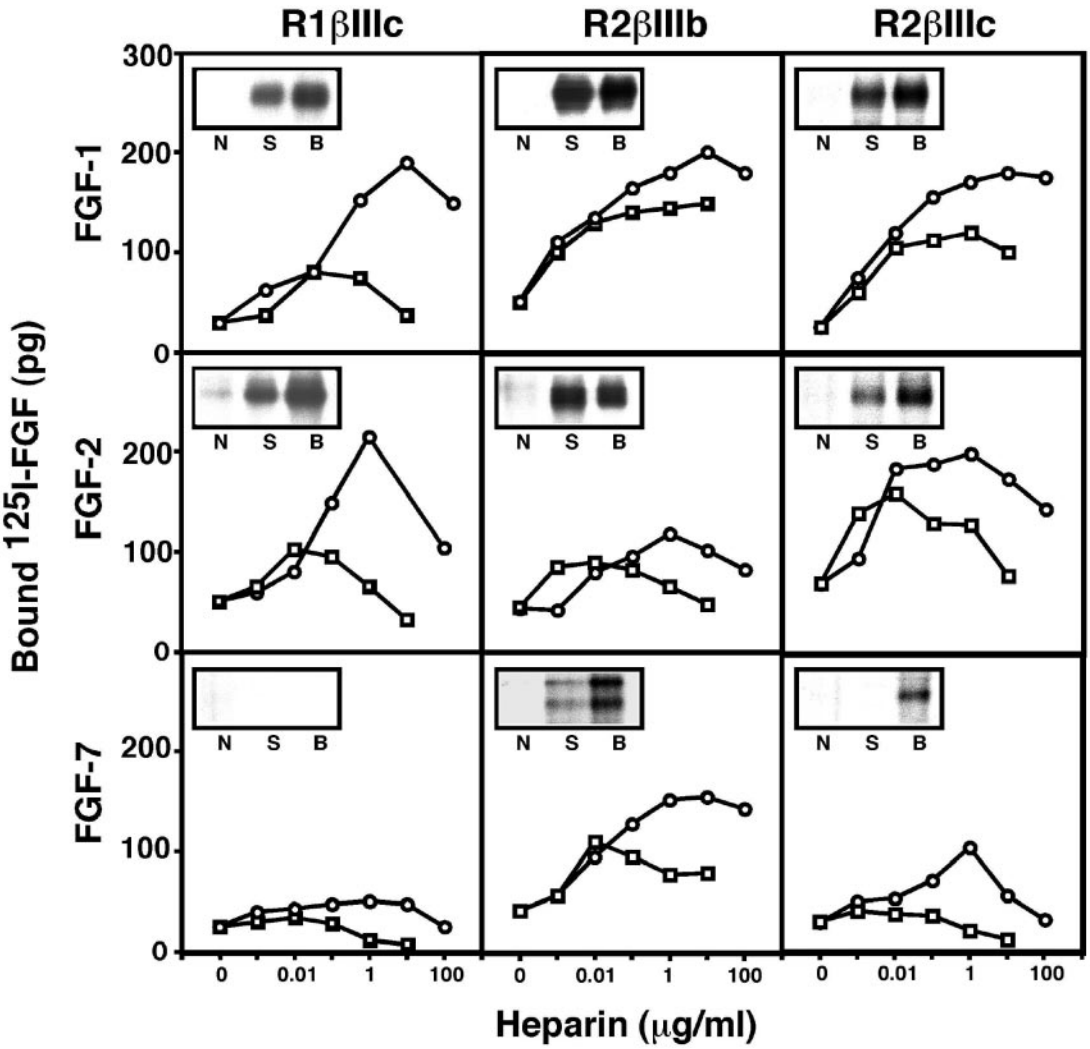

FIG. 2. Effect of soluble heparin or heparin complexed to intact FGFR1β and FGFR2β on the binding of ^{125}I-FGF. The indicated recombinant constructions were prepared and immobilized on GSH–Sepharose beads and incubated with the indicated amounts of heparin followed by removal of unbound heparin as described under Materials and Methods. The binary complexes were then introduced into binding assays containing the indicated radiolabeled FGF. Amount of ^{125}I-FGF bound to the binary complexes (circles) was compared to that bound in assays containing the indicated amounts of soluble heparin (squares). Insets: Radiolabeled complexes formed after covalent affinity crosslinking in the absence of heparin (N), in the presence of 1 μg/ml of soluble heparin (S), or to the binary complexes (B) after removal of unbound heparin. The data points are the mean of duplicate assays, which varied less than 10% among duplicates. The indicated data for each construct is from a single representative experiment, which was reproduced at least three times. All constructs except FGFR2βIIIb with FGF-7 exhibited a single radiolabeled band that correlated with molecular weight of the insect cell derived product plus one FGF. FGFR2βIIIb exhibited two radiolabeled species, which differed by 23 kDa, the molecular weight of the ^{125}I-FGF-7.

for FGF-7 than a chimera comprised of FGFR1 module II and the FGFR2 exon IIIb.

Binding of FGF-1, FGF-2 and FGF-7 to binary complexes of heparin and isolated Ig modules IIIb and IIIc of FGFR2. Previous attempts to demonstrate the binding of FGF-1, FGF-2 and FGF-7 to a number of constructs of variants of Ig module III of FGFR1 and FGFR2 with diverse flanking sequences in conventional binding assays containing soluble heparin failed (4, 6). Therefore, the binding of FGF-1, FGF-2 and FGF-7 cDNA constructs coding for five residues of the FGFR2 amino terminus, the inter-Ig module II-III domain and Ig modules IIIb or IIIc (Fig. 1) was examined under the two conditions described above for module II. The amino terminus of this experimental construct necessarily required the secretory signal of FGFR2 which when processed left five residues of the mature FGFR2 amino terminus. The aminoterminal boundary of the experimental module was arbitrarily defined at the beginning of the exon coding for the inter-module II-III sequence and module III which has subsequently been defined

TABLE 1

Dissociation Constants (K_d values) of FGFR Modules for FGF-1, FGF-2, and FGF-7

	$K_d \pm$ SE (pM)		
Construct	FGF-1	FGF-2	FGF-7
R2βIIIb	97 ± 23	74 ± 20	199 ± 22
R2βIIIc	59 ± 12	104 ± 11	342 ± 82
R2L2	582 ± 54	86 ± 18	70 ± 20
R2L3IIIb	ND	80 ± 21	69 ± 19
R2L3IIIc	ND	165 ± 29	418 ± 43
R1βIIIc	182 ± 13	714 ± 138	ND
R1L2	158 ± 33	294 ± 58	ND

Note. K_d values were calculated from Scatchard analysis as described under Materials and Methods. The values indicated are from one of three experiments. SE, standard error; ND, not detectable, binding was too low to estimate a K_d.

in crystal structures (12, 13). Both isoforms of module III failed to detectably bind FGF-1 independent of binding assay conditions and mode of introduction of heparin (Fig. 3, upper panel). In contrast, both isoforms of module III bound both FGF-2 and FGF-7 with the apparent K_ds of less than 1 nM indicated by Scatchard analysis (Table 1). Covalent crosslinking confirmed the interaction of radiolabeled FGF-2 and FGF-7 with the module III constructs (Fig. 3). The binding of both FGF-2 and FGF-7 to the isolated modules was completely dependent on the introduction of heparin as a complex formed by preincubation and removal of soluble heparin (Fig. 3). Separate results not shown here suggest that the short inter-module sequence (ERSPHRP) beginning at the amino terminus of module III defined by crystal structures does not affect the described binding profile.

These results confirm those of Cheon *et al.* (5) which showed that FGFR2 module IIIb that was fused with an Ig heavy chain at its COOH-terminus bound FGF-7, but failed to bind FGF-1. Our results further suggest that heparin may interact with Ig module III in addition to the primary heparin-binding site on module II. This is consistent with our previous reports which showed that heparin protects a fragment of the FGFR2β ectodomain from tryptic cleavage which extended through the inter-Ig module sequence and into Ig module III (4, 14).

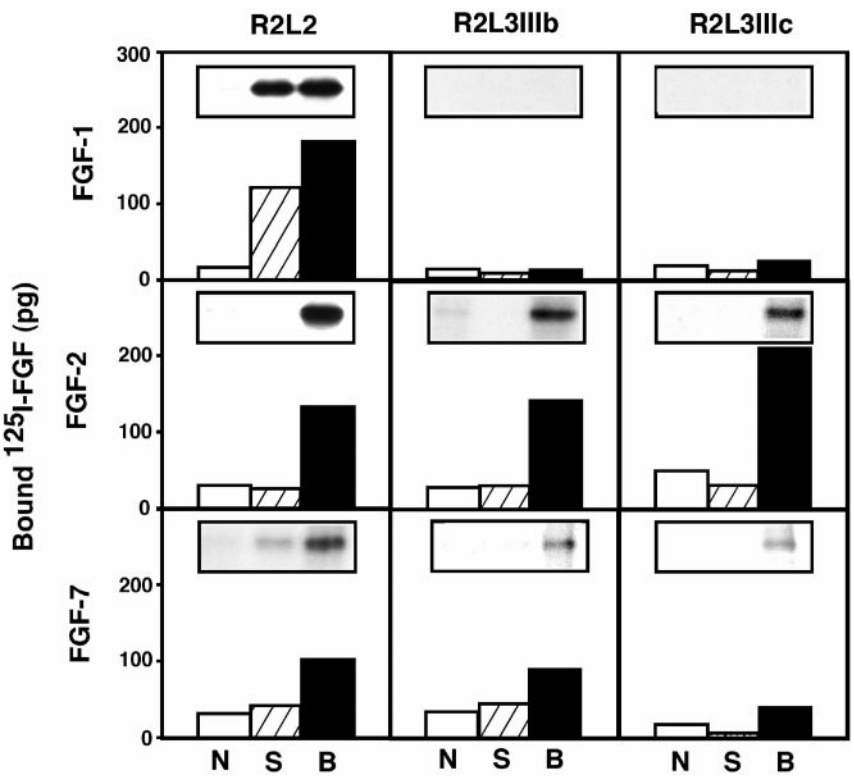

FIG. 3. ^{125}I-FGF binding to Ig modules of FGFR2. The indicated modular constructs of FGFR2 (Fig. 1) were immobilized on GST-beads. ^{125}I-FGF binding to the indicated modules was performed in the absence of heparin (N), in the presence of 1 μg/ml soluble heparin (S), or after incubation of the FGFR modules with 1 μg/ml heparin followed by removal of unbound heparin and washing (B) as described under Materials and Methods. The data are the mean of duplicates and representative of three independent experiments performed on each construct. The insets show the covalent crosslinked radiolabeled species from each condition.

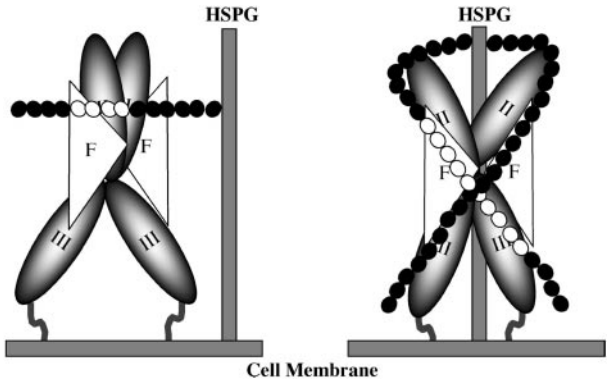

FIG. 4. Models of an oligomeric heparan sulfate–FGFR kinase complex. Model 1 is the one proposed from current crystal structures of the FGF–FGFR complex (12, 13). A single heparan sulfate chain from a proteoglycan core is proposed to run through a channel spanning FGF ligands on each partner of the dimer and the interaction of the heparin-binding domains of two adjacent Ig module IIs which make contact. Each FGF contacts both FGFR. Model II is a modification of our previous model (1) that was generally confirmed by the crystal structures. A single heparan sulfate chain may contact the heparin-binding site on module II of one FGFR, the inter-Ig loop connector sequence and module III on the same FGFR, and extend to Ig module III on the adjacent FGFR. Part of the same chain may contribute to one FGF binding site on module II of one FGFR and another part a separate FGF binding site on module III of the adjacent partner. Beads depict the heparan sulfate chain. White beads indicate parts of the chain that runs behind the indicated Ig module or FGF.

Conclusions. The current results suggest a revision of current models and have implications on the specificity of FGF and the stepwise mode of activation of the complex by FGF (Fig. 4). First, the results suggest that heparan sulfate interacts with module III in addition to the primary binding domain on module II. From the crystallographic structures of the dimeric FGF-FGFR complex, it has been suggested that a single heparan sulfate chain may run across a basically charged channel formed by the heparin-binding domains of both Ig module II units in the dimer while contacting both FGFs (12, Fig. 4). From the current results, we propose that a heparan sulfate chain bound to module II and bridging the FGF in one FGF-FGFR partner may also interact with module III. This interaction has potential to impose restrictions on the conformational relationship between modules II and III, not only *in cis* mode within an FGFR monomer, but *in trans* mode across the dimer (Fig. 4). Second, our results suggest that Ig module II and III, when complexed with affinity selected heparin exhibit separate, distinct binding sites for FGF which is consistent with the proposals of Cheon *et al.* (5) and Chellaiah *et al.* (15). Stoichiometric analysis in solution and FGF-FGFR crystal structures FGFR (12, 13) suggest that it is unlikely that monomeric FGFRβ can accommodate two FGFs at the same time. However, our results suggest that asymmetric binding of FGF-2 or FGF-7 to module II or III on one partner of a dimer and module III or II, respectively, on the other partner may occur. Whether the distinct modular sites cooperate across the dimer in a stepwise mode as proposed by Chellaiah *et al.* (15) remains to be established. A single heparan sulfate chain with properly spaced module- and FGF-specific motifs and the requirement for binding to two distinct sites across the dimer with different determinants of specificity together would impose a high degree of specificity for FGF-2 and FGF-7 within the dimeric complex (Fig. 4). Finally, each asymmetrically bound FGF must also be compatible in *cis* with the other Ig module within the monomer as suggested in previous models (4, 7) and crystal structures (12, 13) This further contributes to the specificity of the overall complex for activating FGF. Consistent with its relative promiscuity *in vitro* with heparin as co-factor, the binding determinants for FGF-1 appear predominantly on the module II complex with little involvement of module III determinants. Therefore, specificity for FGF-1 will be determined by cell-specific heparan sulfate (8) rather than determinants encoded in the FGFR modules.

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