Mapping Ligand Binding Domains in Chimeric Fibroblast Growth Factor Receptor Molecules

MUTIPLE REGIONS DETERMINE LIGAND BINDING SPECIFICITY

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Fibroblast growth factors (FGFs) mediate essential cellular functions by activating one of four alternatively spliced FGF receptors (FGFRs). To determine the mechanism regulating ligand binding affinity and specificity, soluble FGFR1 and FGFR3 binding domains were compared for activity. FGFR1 bound well to FGF2 but poorly to FGF8 and FGF9. In contrast, FGFR3 bound well to FGF8 and FGF9 but poorly to FGF2. The differential ligand binding specificity of these two receptors was exploited to map specific ligand binding regions in mutant and chimeric receptor molecules. Deletion of immunoglobulin-like (Ig) domain I did not effect ligand binding, thus localizing the binding region(s) to the distal two Ig domains. Mapping studies identified two regions that contribute to FGF binding. Additionally, FGF2 binding showed positive cooperativity, suggesting the presence of two binding sites on a single FGFR or two interacting sites on an FGFR dimer. Analysis of FGF8 and FGF9 binding to chimeric receptors showed that a broad region spanning Ig domain II and sequences further N-terminal determines binding specificity for these ligands. These data demonstrate that multiple regions of the FGFR regulate ligand binding specificity and that these regions are distinct with respect to different members of the FGF family.

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‡ The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; AP, alkaline phosphatase; PCR, polymerase chain reaction.
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Fibroblast growth factors (FGFs) are essential signaling molecules that regulate embryonic growth, development, cell proliferation, differentiation, and angiogenesis (1). At least 19 related members of the FGF family have been identified thus far, and all but four are known to activate one of four high affinity cell surface FGF receptors (FGFR) (2–9). The FGFRs consist of an extracellular region containing three immunoglobulin-like (Ig) domains, a stretch of seven conserved acidic amino acids, and a heparin binding domain (10, 11). The Ig domain III is clearly important for ligand binding and shows specificity toward different ligands. For example, specific mutations in this region in FGFR2 can decrease the binding of FGF2 without affecting the binding of FGF1 or FGF7 (42). The b splice form of FGFR3 (FGFR3b) also has unique properties in that it can only be activated by FGF1, which shows little specificity toward any receptor, and FGF9, which shows no activity toward FGFR1b and FGFR2b (21, 43, 44). Alternative splicing of Ig domain III can also lead to truncation of the transmembrane and intracellular regions ("a" splice form) creating a secreted FGF-binding protein (45). The physiological relevance of this form of the receptor is not known; however, recent studies demonstrated that in transgenic mice overexpression of a soluble FGFR extracellular domain can result in dramatic developmental defects (46). Another major alternative splicing event truncates Ig domain I. Receptor forms lacking Ig domain I have a higher affinity for some FGF ligands, although it is not known if ligand binding specificity is affected by this splicing event (47–49). Truncation of Ig domain I also correlates with the progression of several tumors toward malignancy, suggesting a functional difference between long and short receptors (50, 51).
In addition to alternative splicing, differential binding specificity also exists when similarly spliced receptors are compared. For example, FGFR8, FGFR17, and FGFR18 can activate FGFR2c and FGFR3c but show little activity toward FGFR1c (21, 39). FGFR6 can activate FGFR1c and FGFR2c but shows little activity toward FGFR3c (21, 52). Although much is known about how alternative splicing affects ligand binding specificity, the molecular basis of binding specificity between similarly spliced receptors is not known. In this study we examine determinants of ligand binding specificity by comparing the binding activity of FGFR1c and FGFR3c. We have generated a series of soluble chimeric receptors and have assayed these molecules for binding to FGFR1, FGFR2, FGFR8, and FGFR9. These data demonstrate that the FGFR contains two ligand binding regions. FGFR1 binds either region in either receptor, FGFR2 preferentially recognizes distal sequence (Ig domain II–III) of FGFR1, FGFR8 preferentially recognizes sequences both N-terminal and C-terminal to Ig domain II of FGFR3, and surprisingly, FGFR9 binding specificity is only dependent on sequences N-terminal to and including Ig domain II in FGFR3 with no preference for the linker region between Ig domain II and III or sequence in the alternatively spliced region of Ig domain III.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant FGFR1 was a gift from Merck; human recombinant FGFR2 was a gift from Novo Novartis; mouse recombinant FGFR9 (44) was obtained from PeproTech Inc. Heparin was obtained from Duke University. Mouse recombinant FGF8b was a gift from C. MacArthur (53); mouse recombinant FGF1, FGF2, FGF8, and FGF9 were used in the binding studies within 48 h of preparation. For example, FGF8, FGF17, and FGF18 can activate FGF Receptor Binding Specificity.

Construction of Chimeric Receptor Molecules—Solving chimeras are defined as replacements of internal regions of one receptor with that of another. Solving chimeric receptors 3Sa, 3Sb, 3Sc, 3Sd, and 3Se have the backbone sequence of FGFR3, whereas 1Sa and 1Sb have the backbone sequence from FGFR1. Two pairs of internal primers were used to assemble each chimeric receptor. The 5' and the 3' junctions are indicated as follows: 3Sa, (5'-sense) 5'-TTCCGCGCAGTGGTACGTCAGTC-3' and 3Sb, (5'-antisense) 5'-CTATACCTGTATGTTGAGAACAAG-3'; 3Sc, (5'-sense) 5'-CTATACCTGTATGTTGAGAACAAG-3' and 3Sd, (5'-antisense) 5'-CAACTACGTTGTTGAGAACAAG-3'; 3Sf, (5'-antisense) 5'-CTATACCTGTATGTTGAGAACAAG-3'; 3Sg, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sh, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Si, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sj, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sf, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sc, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sa, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sb, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sc, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sd, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Se, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sf, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sg, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sh, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Si, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sj, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sf, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sg, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sh, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Si, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sj, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sf, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sg, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sh, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Si, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'; 3Sj, (5'-sense) 5'-AGGAACGGCCGAGGACGAGC-3'.

Point Mutation of FGFR3—Point mutations, introduced into the C terminus of FGFR3 Ig domain III (constructs C-13, C-14, C-15, and C-16), were engineered using PCR amplification and appropriate mutant oligonucleotide primers. All mutations were confirmed by DNA sequencing.

Production of FGFR-AP Conditional Media—Cos-7 cells were transfected by the DEAE-dextran method (56) with 20 µg of plasmid/106 cells. Conditioned media were collected after 3 days and replaced with fresh media. Subsequently, media were collected every 2 days over a period of 9 days. The conditioned media were assayed for AP enzyme activity to normalize for receptor number in binding assays (55). AP enzyme activity was determined by transferring 50 µl of conditioned media to a flat bottom microtiter plate and adding 50 µl of a 2X AP assay solution (2 ml diethanolamine, 1 mM MgCl2, 20 mM hemoglobin, 12 mM p-nitrophenol phosphate (Sigma)) and measuring the change in optical density at 405 nm in a kinetic microtiter plate reader ( Molecular Devices Inc.).

Binding Assays—FGFR1, 2, and 3 were iodinated using the chloramine T method to a specific activity of 800–30000 CPM/nmol as described previously (57). 1–3 µg of FGF was incubated with 1 µCi of Na125I (Amersham Pharmacia Biotech), 45 µg/ml chloramine T (Eastman Kodak Inc.), and 143 mM HEPES, pH 7.4, in a volume of 70 µl for 2 min at 23°C. 100 µl of 20 mM dithiorthiol was then added, and the mixture was then incubated for an additional 10 min. The mixture was then applied to a heparin–agarose (200 µl, 2 ml/ml heparin) column that had been equilibrated with 20 mM HEPES, pH 7.4, 0.2% bovine serum albumin, and 0.4 mM NaCl. Labeled growth factor was eluted with 20 µl of HEPES, pH 7.4, 0.2% bovine serum albumin, and 3 mM NaCl. Labeled FGFR1 and FGFR9 were used in the binding studies within 48 h of labeling. FGFR2 was used up to 2 weeks after labeling. The labeled growth factors were stored at -70°C. Binding assays were set up by adding components at 4°C in the following order: Dulbecco’s modified Eagle’s medium to a flat bottom microtiter plate and adding 50 µl of conditioned media to a flat bottom microtiter plate and adding 50 µl of a 2X AP assay solution (2 ml diethanolamine, 1 mM MgCl2, 20 mM hemoglobin, 12 mM p-nitrophenol phosphate (Sigma)) and measuring the change in optical density at 405 nm in a kinetic microtiter plate reader (Molecular Devices Inc.).
**FGF Receptor Binding Specificity**

FIG. 1. Primary structure of mouse FGFR1 and FGFR3 extracellular domains. Top, schematic diagram showing the various regions of the FGFR defined in the sequence alignment below. Bottom, sequence comparison of mouse FGFR1 and FGFR3 extracellular domains. Identical amino acid residues are shaded. Lowercase letters in the transmembrane (TM) domain indicate sequence replaced by alkaline phosphatase (AP) in the generation of soluble FGFR extracellular domains. The bold underline indicates sequence deleted (N terminus deletion) to construct 2 Ig domain forms of FGFR1 (top) and FGFR3 (bottom). Defined regions of the extracellular domain are labeled and indicated with arrows. Signal peptide (SP), N terminus (A); immunoglobulin-like loops (Ig loop I, II, and III); I–II linker sequence (B); II–III linker sequence (C); juxta-transmembrane region (J); transmembrane domain (TM). The open box in the schematic at the top of the figure represents the highly conserved stretch of acidic residues in the I–II linker.

Eagle’s medium (Life Technologies, Inc.) with 0.1% bovine serum albumin; 30 μl of a 2% slurry of anti-alkaline phosphatase monoclonal antibodies coupled to Sepharose (55); 10 μl of 25 μg/ml heparin; FGFR-AP conditioned media containing specific soluble FGFRs (300 optical density units of AP activity) (54); unlabeled FGF as a competitor (up to a concentration of 80 nM or approximately an 800-fold molar excess); and 125I-FGF (30,000–50,000 cpm) in a volume of 250 μl. The reaction was gently rotated for 120 min at 4 °C. Bound receptor and FGF were recovered by centrifugation (10 s at 12,000 rpm (4000 g); 4 °C in a microcentrifuge). Free ligand concentration was determined by dividing the measured cpm bound or cpm free by the calculated specific activity (specific activity times dilution factor). Binding curves were then calculated and fit to either a two-ligand binding equation (58, 59) to calculate binding constants. The signal peptide (SP), N terminus (A), and Ig loop I, II, and III (shaded) are identical between FGFR1 and FGFR3 to minimize nonspecific effects on protein structure. All molecules were tagged at the C terminus with human placental AP, which serves as an enzymatic marker to normalize protein concentration and as an epitope tag for immunoprecipitation binding assays.

RESULTS

Sequence comparison of the FGFR1 and FGFR3 extracellular regions (c splice form) shows a high degree of similarity and several highly conserved regions of identity (Fig. 1). The most highly conserved sequences are in Ig loop II, the linker region between Ig loop II and III (II–III linker region) (C), Ig loop III and through the N-terminal part of the juxta-transmembrane region (J). The signal peptide (SP), N terminus (A), and Ig loop I are less well conserved, and the I–II linker (B) shows some regions that are highly conserved. For the purposes of this study the Ig loop I is defined as the sequence between the conserved disulfide-linked cysteine residues within each Ig domain. To examine the molecular basis for ligand binding specificity, deletions, chimeric molecules, and scanning mutations were constructed throughout the extracellular domains of FGFR1 and FGFR3. Fusion sites were chosen at residues that are identical between FGFR1 and FGFR3 to minimize nonspecific effects on protein structure. All molecules were tagged at the C terminus with human placental AP, which serves as an enzymatic marker to normalize protein concentration and as an epitope tag for immunoprecipitation binding assays.

**Ligand Binding Specificity of FGFR1 and FGFR3 Molecules Lacking Ig Domain I**—The binding activity of the full-length FGFR1 extracellular domain was compared with a receptor in which the N-terminal Ig domain was deleted (FGFR1Δ1). The deletion in the N terminus corresponded to that of a naturally occurring variant in which Ig domain I is skipped by altern-
three and two Ig domain FGFRs. FGFR1 and FGFR3 require the presence of heparin for FGF2 binding to open bars. Binding assays were carried out in the presence (+, solid) or absence (−, shaded) of 50 ng/ml heparin. C, binding of iodinated FGF1, FGF2, and FGF9 to three and two Ig domain forms of FGFR1 (solid) and FGFR3 (open). COS, binding to conditioned media from untransfected COS-7 cells.

**FIG. 2.** Ligand binding specificity of three and two Ig domain FGFRs. A, schematic diagram showing the three (FGFR1 and FGFR3) and two (FGFR1Δ and FGFR3Δ) Ig domain forms of the FGFR as indicated. FGFR1 sequence is compared with FGFR3, and FGF9 bound both long and short forms of FGFR3 but showed no binding to either form of FGFR1 over background levels (Fig. 2C). These data demonstrated that the N-terminal Ig domain is not a determinant of ligand binding specificity and therefore localized the binding domain(s) to regions encompassing Ig domain II and III. To localize further receptor regions that determine binding specificity, a series of chimeric molecules were constructed throughout the extracellular sequences (B, II, C, III and J; defined in Fig. 1) of FGFR1 and FGFR3 and assayed for ligand binding.

Binding Properties of Soluble Chimeric FGFR Receptors—To map regions that determine ligand binding specificity, two types of receptor mutations were constructed (Fig. 3). 1) Chimeric receptors, named 1,3 or 3,1 (Fig. 3B), consist of a simple fusion between FGFR1 and FGFR3 at sites of amino acid identity such as conserved cysteine residues. 2) Scanning mutations, named 1S or 3S (Fig. 3C), have a backbone structure from FGFR1 or FGFR3, respectively, and contain sub-regions derived from the other receptor, again fused at sites of amino acid identity.

Because mitogenic assays assessing ligand-receptor specificity demonstrated that FGF1 is the only member of the FGF family that can activate all receptors, regardless of splice form (21), the ability of FGF1 to bind each chimeric receptor was tested. All chimeric receptors bound FGF1 significantly over background (Fig. 3). Only two scanning mutations, 3Sa and 1Sa, showed 2-fold lower binding compared with most other molecules. These data demonstrate that the primary structure of the chimeric receptors is not significantly altered and that these chimeric molecules are therefore suitable for testing the specificity of other potentially more selective ligands.

In all the binding studies shown, the quantity of soluble receptor used was normalized to AP enzyme activity. To demonstrate that AP activity correlates with the amount of protein present, several of the preparations were also compared by Western blot analysis. Immunoprecipitation of 40 units of AP activity, followed by Western blot and detection with anti-AP monoclonal antibodies (Fig. 4A), showed similar band intensities and confirmed that enzymatic quantification of AP activity is a good measure of receptor protein concentration. Additionally, the predicted 10-kDa deletion in FGFR1ΔAP is demonstrated by directly analyzing both FGFR1AP and FGFR1ΔAP conditioned media by Western blot without prior immuno-
FGFRs. Top left (top lane) measurements are plotted for each chimeric and chimeric receptors diagrammed in FGF1 to the corresponding panel of 3S bone sequence derived from FGFR3 (Right, 1S). FGFR3 sequence is fused to FGFR1 sequence derived from FGFR3 (chimeric FGFRs in which FGFR1 sequence is fused to FGFR3). Open bars indicate sequence derived from FGFR3. Solid bars defined in Fig. 1. A–C. Regions of the FGFR are colored as in Fig. 5. Western blot analysis of the soluble FGFR-AP fusion proteins. A, 40 units (see Experimental Procedures) of the indicated soluble FGFR-AP proteins were immunoprecipitated using wheat germ agglutinin-Sepharose, separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto a polyvinylidene difluoride membrane. The blots were hybridized with anti-AP antibody, and bands were visualized with a peroxidase-conjugated secondary antibody and ECL detection. The positions of molecular mass markers are indicated on the right. B, 20 units of FGFR1-AP and FGFR1Δ-AP conditioned media were directly loaded onto a 6% SDS-polyacrylamide electrophoresis gel that was electroblotted and probed with anti-AP antibody as described above.

Specificity of Chimeric Receptors for FGF2—FGF2 consistently binds FGFR1 to a greater extent than FGFR3 (Fig. 2, see Refs. 15 and 54) but nevertheless can similarly activate either receptor in a mitogenic assay (21). The FGFR extracellular sequence(s) responsible for this differential binding were mapped by analysis of FGF2 binding to the panel of chimeric receptor molecules shown in Fig. 3.

Chimeric receptors containing sequence from the N terminus of FGFR1, fused to the C terminus of the FGFR3 extracellular region (1,3-a, 1,3-b, 1,3-c), bind less FGF2 than full-length FGFR3 (Fig. 5, A and B) but similar amounts of FGF1 (Fig. 3B). These three molecules all have the FGFR3 Ig loop III and J region in place of FGFR1. In contrast, when Ig loop III is derived from FGFR1 (1,3-d and 3Se, Fig. 5) binding is similar to that of full-length FGFR1. Chimeric molecules containing the N terminus of FGFR3 and progressively more FGF1 sequence from the C terminus bind FGF2 as well (3,1-b) or better (3,1-c, 3,1-d, and 3,1-e) than full-length FGF1 once the terminal 30 amino acid residues of Ig loop III are derived from FGFR1 (compare 3,1-a to 3,1-b or 3,1-c in Fig. 5B). These data demonstrate that the C-terminal half of FGFR1 Ig domain III ( exon c) is important for FGF2 binding and that the I—II linker region of FGFR1 may inhibit FGF2 binding. Consistent with this, scanning mutants in which small mutations of FGFR3 are replaced by corresponding sequence from FGFR1 bind FGF2 less well than full-length FGFR1 if the sequences exclude the

### Table I

| FGFR extracellular domain | $K_a$ | $K_d$ | $X_m$ | $n_{1/2}$ | $K_d$ |
|---------------------------|-------|-------|-------|-----------|-------|
| FGFR1                      | 7.2 ± 2.0 x 10^a | 1.7 ± 0.6 x 10^b | 2.9 ± 0.7 x 10^-10 | 1.7 ± 0.1 |
| FGFR1Δ                     | 1.5 ± 0.2 x 10^9 | 1.4 ± 0.3 x 10^8 | 2.2 ± 0.3 x 10^-10 | 1.5 ± 0.1 |
| FGFR 1Sa                   | 4.7 ± 2.2 x 10^8 | 2.8 ± 1.6 x 10^7 | 2.8 ± 1.0 x 10^-10 | 1.8 ± 0.1 |
| FGFR 1Sb                   | 3.6 ± 2.4 x 10^10 | 1.7 ± 1.2 x 10^9 | 4.1 ± 2.4 x 10^-11 | 0.8 ± 0.3 |
| FGFR 3,1-c                 | 4.1 ± 0.3 x 10^9 | 5.1 ± 2.0 x 10^8 | 2.2 ± 0.4 x 10^-10 | 1.1 ± 0.1 |
| FGFR 3,1-e                 | 1.4 ± 0.7 x 10^10 | 6.0 ± 4.3 x 10^9 | 3.5 ± 1.5 x 10^-11 | 1.3 ± 0.2 |

* Binding constants for a two-ligand binding reaction.
* $X_m$, mean ligand activity, defined as $(K_iK_d)^{-1/2}$, for a two-ligand binding equation.
* $n_{1/2}$, Hill coefficient, defined as $2/(1 + K_d/K_i)^{1/2}$.
* $K_d$, dissociation constant for a single ligand binding reaction.
* Error indicates the 95% confidence level derived from the least squares analysis.

**FIG. 3. FGF1 binding to chimeric FGFRs.** Top left, structural features of the FGFR extracellular domain in relation to map positions of chimeric FGFRs shown in A–C. Regions of the FGFR are colored as in Fig. 5. Solid bars indicate sequence derived from FGFR1. Open bars indicate sequence derived from FGFR3. A, three Ig domain FGFR-AP molecules used as controls in all experiments. B, chimeric FGFRs in which FGFR1 sequence is fused to FGFR3 (1,3-a) or in which FGFR3 sequence is fused to FGFR1 (3,1-b). C, scanning mutants with a backbone sequence derived from FGFR3 (3S) or FGFR1 (1S). Right, binding of indicated FGFR1 to the corresponding panel of chimeric receptors diagrammed in A–C. The mean cpm bound (shaded bars) and standard deviations of duplicate measurements are plotted for each chimeric FGFR and for control conditioned media (top lane in A).

**FIG. 4. Western blot analysis of the soluble FGFR-AP fusion proteins.** A, 40 units (see Experimental Procedures) of the indicated soluble FGFR-AP proteins were immunoprecipitated using wheat germ agglutinin-Sepharose, separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto a polyvinylidene difluoride membrane. The blots were hybridized with anti-AP antibody, and bands were visualized with a peroxidase-conjugated secondary antibody and ECL detection. The positions of molecular mass markers are indicated on the right. B, 20 units of FGFR1-AP and FGFR1Δ-AP conditioned media were directly loaded onto a 6% SDS-polyacrylamide electrophoresis gel that was electroblotted and probed with anti-AP antibody as described above.
C-terminal 30 amino acid residues of FGFR1 Ig loop III (3Sa to 3Sd). However, scanning mutations containing these 30 residues from FGFR1 bind FGF2 at least as well as full-length FGFR1 (3Se to 3Sg), again demonstrating the importance of this sequence.

Within the C-terminal 30 amino acid residues of Ig loop III, five differences exist between FGFR1 and FGFR3 (Fig. 6A). To determine whether single or multiple amino acid residues account for these differences, residues in FGFR3 were sequentially changed to residues used in FGFR1, and FGF2 binding was assayed. The H327R, S325H, or the double mutation (H327R/S325H) resulted in only a small increase in FGF2 binding (Fig. 6B). Adding a third point mutation, L321M (H327R/S325H/L321M) significantly enhanced FGF2 binding. However, levels of FGF2 binding comparable to that of FGFR1 were not attained until all five residues were changed (3SG, Figs. 5C and 6). These data suggest that the sequence context of this entire region is required for optimal binding of FGF2 and that no single amino acid difference can account for the decreased binding to FGFR3.

Several of the chimeric receptors consistently bind more FGF2 than the full-length FGFR1 extracellular domain (3,1-c, 3,1-d, 3,1-e, 3Se, and 1Sa in Fig. 5). These “activated” molecules all have in common sequences derived from the FGFR3 linker region between Ig loops I and II (I—II linker (B) in Fig. 1) and FGFR1 sequence derived from Ig loop III. These data suggest that the I—II linker region in FGFR3 may contribute to a second FGF2 binding region that, together with a primary binding region localized in FGFR1 Ig loop II—III, increases the FGF2 binding capacity. Alternatively, because FGFR1 already has two linked binding regions (see below), uncoupling these regions in chimeric receptors may increase binding by relieving constraints on the individual regions. The N-terminal binding region in FGFR3, along with an inactive FGFR3 Ig loop III binding region, may account for the decreased FGF2 binding observed in the full-length FGFR3 extracellular domain compared with the FGFR1 extracellular domain. To examine further these possibilities, the number of binding sites and binding affinity of chimeric receptor molecules were assessed.

The binding affinities of FGFR1, FGFR1Δ, and chimeric receptors 1Sa, 1Sb, 3,1-c, and 3,1-e, to FGF2, were determined by competition for binding of iodinated FGF2 with increasing amounts of unlabeled FGF2 up to an 800-fold molar excess (Fig. 7 and data not shown). Interestingly, Scatchard plots (B/F versus B) of FGFR1, FGFR1Δ, and 1Sb showed a concave curve instead of a straight line (Fig. 7, A—C, right) suggesting the presence of more than one interacting binding region in FGFR1 (62, 63). In contrast, chimeric receptors 1Sa, 3,1-c, and 3,1-e (which showed increased binding capacity) have linear Scatchard plots (Fig. 7, D—F, right) indicating single or identical and
to either a single site binding equation ($B = B_{\text{max}} F(K_d + F)$; $B = \text{bound ligand}; F = \text{free ligand}$) or the two-site equation (Table I). The Hill coefficients for these receptor binding domains are close to 1 providing further evidence for single or identical and independent binding sites in these chimeric molecules.

**Specificity of Chimeric Receptors for FGFR8 and FGFR9**—FGFR8 and FGFR9 can bind and activate FGFR3c but not FGFR1c. Furthermore, unlike all other FGFs tested, only FGFR1 and FGFR9 can bind and activate FGFR3b (Refs. 21, 39, 43, and 44 and Fig. 2). Comparison of FGFR8 and FGFR9 activity shows that both ligands activate FGFR3c but not FGFR1c, but unlike FGFR9, FGFR8 cannot interact with FGFR3b. To evaluate the molecular basis for this specificity the ability of FGFR8 and FGFR9 to interact with chimeric receptors was assayed.

FGR9 showed no binding over background levels to chimeras containing N-terminal sequences from FGFR1 and C-terminal sequence from FGFR3 (1,3-a to 1,3-d, Fig. 8). However, FGFR9 bound well to all chimeric receptors containing N-terminal sequence from FGFR3 that included Ig loop II (1,3-a to 3,1-d, Fig. 8). All chimeras containing either Ig loop II sequence derived from FGFR1 (3,1-e, 3Sa, and 1Sa, Fig. 8) or I–II linker sequence derived from FGFR1 (1,3-a to 1,3-d, 1Sb, Fig. 8) did not bind FGFR9. Whereas all chimeric receptors containing both the FGFR3 I–II linker and FGFR3 Ig loop II bound FGFR9 at levels equal to or greater than that of full-length FGFR3 (3,1-a to 3,1-d, 3Sb to 3Sg, Fig. 8).

Because FGFR8 and FGFR9 have similar binding specificity with respect to FGFR3c and FGFR1c but discordant activity toward FGFR3b, the receptor binding regions for these two ligands were compared. The ability of FGFR9 (which cannot be efficiently iodinated) to compete with 125I-FGFR1 binding to chimeric receptors was assayed. FGFR1 was used because of its ability to activate all splice forms of all FGFRs and to bind all chimeric FGFRs (Ref. 21, Fig. 3). As predicted from the known specificity of FGFR8 (39), it could compete efficiently (defined as greater than 10% competition) for binding to FGFR3 but not to FGFR1 (Fig. 9A). FGFR8 failed to compete for binding to any chimeric receptor that contained FGFR1 derived sequence from the I–II linker, Ig loop II, or the N terminal of the II–III linker (1,3-a to 1,3-d, 3,1-d, 3,1-e, 3Sa, and 3Sc, Fig. 9B). Significantly, FGFR9 showed a similar binding profile to that of FGFR8 except for chimeras 3,1-d and 3Sc, molecules that bound FGFR9 well but did not bind FGFR8. This difference in binding suggests that the I–II linker, Ig loop II, and the N-terminal half of the II–III linker of FGFR3 are all important for FGFR8 binding, whereas only the I–II linker and Ig loop II specify FGFR9 binding. FGFR8 bound poorly to chimeras 3,1-b, 3,1-c, and 3,1-d, and 3Sc compared with full-length FGFR3, 3,1-a, and 3Sd. Chimeras 3,1-b, 3,1-c, 3,1-d, and 3Sd have in common FGFR1 sequence from Ig loop III suggesting that this region of FGFR1 is also unfavorable for FGFR8 binding.

**DISCUSSION**

FGFs bind and activate a subset of the four known FGFRs. One well described mechanism that modulates the binding specificity of an individual FGFR is alternative splicing in Ig loop III (12–16, 18–20). However, sequence differences between FGFRs must also regulate specificity. In this study we have mapped regions of the FGFR that are important determinants of ligand binding specificity of similarly spliced FGFRs. We have also addressed the role of Ig domain I in ligand binding.

Sequence comparison of the extracellular domain of FGFRs 1 and 3 show that the N terminus (region A), Ig loop I, and parts of the I–II linker (region B) are poorly conserved (26% amino acid identity), whereas Ig loop II, the II–III linker (region C), Ig loop III, and parts of the juxta-transmembrane domain (region
J) are highly conserved (66% amino acid identity, Fig. 1). In addition to alternative splicing in Ig loop III, a second common alternative splicing event excludes the exon encoding Ig domain I (region A, Ig loop I, and part of the I–II linker). Comparison of binding to both the three Ig loop form of the receptor and the two Ig loop form showed no difference in binding for FGF1, FGF2, FGF8, or FGF9, suggesting that this region is not a determinant of ligand binding specificity. Furthermore, the affinity and cooperativity of FGF2 binding and the heparin requirement for binding to both long and short receptor splice forms of FGFR1 was similar, indicating that Ig domain I has little effect on the binding affinity of FGF2. These data are in contrast with data of Wang et al. (48) that show that receptors lacking Ig domain I have increased affinity for FGF1 and heparin. It remains possible that Ig domain I is an important determinant of the binding specificity of other members of the FGF family.

To map further the regions within the FGFR extracellular domain that determine ligand binding specificity, the binding of FGF1, FGF2, FGF8, and FGF9 to chimeric molecules between FGFR1c and FGFR3c was assayed. These ligands were chosen because they have distinct differences in their ability to interact with these two receptors. Consistent with mitogenic assays and previous binding assays, FGF1 bound all chimeric molecules significantly over background levels (Refs. 15, 21, and 54 and Fig. 3). Binding assays with FGF2, which preferentially binds FGFR1, shows that FGF2 binds cooperatively to two sites within FGFR1, whereas FGF8 and FGF9 binding is most dependent on a single proximal region within FGFR3 (Fig. 10A). These data suggest that binding specificity is determined by more than one region on an FGFR.

A two binding region model (Fig. 10) is supported by the observed cooperativity of FGF2 binding. This is shown by a concave Scatchard plot and a Hill coefficient of 1.7. Additionally, the binding data fit a two-ligand binding equation much better than a one-ligand binding equation, also supporting the notion of two binding regions. Furthermore, cooperativity is lost in some chimeric molecules suggesting that specific amino acid side chains in the I–II linker and Ig domain II interact with residues in Ig domain III in FGFR1 and that these inter-
molecules binding cooperatively (in cis) to one or two FGFRs if there are two independent and non-interfering binding sites (Fig. 10C, top) or with two FGF2 molecules binding cooperatively to a receptor dimer (in trans) if the binding specificity regions on a single receptor exclude the binding of a second ligand (Fig. 10C, bottom). Interestingly, interaction studies focusing on the highly conserved II–III linker region and a recent crystal structure of FGFR1 complexed with FGF2 (65, 66) demonstrate that receptor dimers can form independently of ligand binding. These studies suggest that cooperative ligand binding could occur in trans to a receptor dimer.

The hypothesis that two FGF binding regions exist within a single FGFR is further supported by studies in which either FGFR2 Ig domain II or Ig domain IIIb, when fused to the immunoglobulin heavy chain constant region, are capable of binding ligand (19). However, the immunoglobulin molecule itself may also contribute to ligand binding so it is still not clear whether an isolated FGFR Ig domain is capable of independently binding ligand. Additionally, a soluble chimeric receptor containing only region B, Ig loop II, and region C of FGFR3 fused to AP failed to bind FGF1 or FGF9 with high affinity (data not shown) suggesting that this region is not sufficient to form an FGF-binding site. Studies by Hou et al. (67) also strongly suggest that both Ig domain II and III are required to form an FGF-binding site and that Ig domain I cannot substitute for either Ig domain II or III.

The presence of a proximal binding region is supported by the observation that the I–II linker sequence and Ig loop II from FGFR3 are both required for FGF9 binding and that the II–III linker sequence and Ig loop III can be derived from either receptor without affecting FGF9 binding specificity (Figs. 8 and 10). The irrelevance of the source of Ig loop III sequence for FGF9 binding provides a molecular explanation for why FGF9 binds to both alternatively spliced forms of FGFR3. However, as stated above, Ig loop III is still required to form an FGF9-binding site because the I–II linker, loop II, and the II–III linker cannot by themselves bind ligand (data not shown). This suggests that there may be interdomain interactions that contribute to the FGF9-binding site.

Exchanging the C-terminal 8 amino acid residues of the J region (chimera 3,1-a), a sequence poorly conserved between FGFR1 and FGFR3, enhanced ligand binding by 1.4- (FGF1) to 2-fold (FGF9). This juxta-transmembrane sequence may therefore contribute to FGF binding or may be involved in orienting the extracellular domain relative to the transmembrane domain or, for the soluble FGFRs examined here, relative to AP.

Unlike FGF8 and FGF9 that show specificity for a region that includes Ig loop II of FGFR3 and FGF2 that recognizes two cooperatively linked binding regions in FGFR1, FGF1 binds all chimeric receptors and can activate any FGFR regardless of alternative splicing (21). This unique property of FGF1 suggests that it can recognize either proximal or distal FGF binding regions derived from any FGFR. FGF8 can compete for up to 45% of the bound 

\[ \text{FGF8} \] to chimeric FGFRs (Fig. 9), whereas FGF1 can displace essentially all bound 

\[ \text{FGF1} \] (data not shown). This observation suggests that FGF8 can only compete at one FGF1 binding region, whereas FGF1 can compete for binding at both regions.

The data presented here and diagrammed in Fig. 10C (top) suggests that there may be two binding sites on a single FGF. In contrast, a recent crystal structure of FGF2 bound to a fragment of FGFR1 shows only one molecule of FGF2 in contact with a pocket formed by Ig domain II, the II–III linker, and Ig domain III (65). This binding pocket corresponds to the distal binding region defined here. However, the fragment of FGFR1 that was crystallized lacks 22 N-terminal amino acids (includ-
ing the highly conserved acidic region) that are present in naturally occurring 2 Ig-domain splice forms of FGFR1 and in FGFR1Δ1 molecule used in this study. It is therefore possible that the fragment used for crystallography has lost a critical component of the proximal binding region. Another possibility that cannot be ruled out is cooperative binding in “trans” across a receptor dimer (Fig. 10C, bottom). Although this model would appear to be more consistent with the FGFR dimer observed in the crystal structure, which defines a binding site that is in close agreement with the distal binding region mapped here, the concentrations of receptor used in the binding assay make it unlikely that such a dimer would form under the conditions of the experiment.

Multiple binding regions in the FGFR may be important to allow four receptors to differentially recognize 19 or more FGF ligands. Additionally, cooperative linkage of multiple binding regions may make FGFR1 responsive to a threshold level of ligand, below which a response is not elicited. This may be an important mechanism regulating FGF2 activity during tissue repair and remodeling. FGF2, which is present on the surface ligand, below which a response is not elicited. This may be an regions may make FGFR1 responsive to a threshold level of the conditions of the experiment.

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