Receptor Specificity of the Fibroblast Growth Factor Family
THE COMPLETE MAMMALIAN FGF FAMILY*

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Fibroblast growth factors (FGFs) are encoded by 22 genes. FGFs bind and activate alternatively spliced forms of four tyrosine kinase FGF receptors (FGFRs 1–4). The spatial and temporal expression patterns of FGFs and FGFRs and the ability of specific ligand-receptor pairs to actively signal are important factors regulating FGF activity in a variety of biological processes. FGF signaling activity is regulated by the binding specificity of ligands and receptors and is modulated by extrinsic cofactors such as heparan sulfate proteoglycans. In previous studies, we have engineered BaF3 cell lines to express the seven principal FGFRs and used these cell lines to determine the receptor binding specificity of FGFs 1–9 by using relative mitogenic activity as the readout. Here we have extended these semiquantitative studies to assess the receptor binding specificity of the remaining FGFs 10–23. This study completes the mitogenesis-based comparison of receptor specificity of the entire FGF family under standard conditions and should help in interpreting and predicting in vivo biological activity.

In mammals, fibroblast growth factors (FGFs) comprise a structurally related family of 22 molecules. FGFs can be grouped into seven subfamilies based on their sequence similarities and functional properties (1–3). FGFs bind four high affinity, ligand-dependent FGF receptor tyrosine kinase molecules (FGFR1–4). In the presence of heparan sulfate (HS) glycosaminoglycans, FGFs stably bind FGFRs and lead to the formation of 2:2 FGF-FGFR-HS dimers, which enables the cytoplasmic kinase domains to transphosphorylate one another and become activated (4). FGFR activation results in the stimulation of various signal transduction cascades that have been implicated in multiple aspects of vertebrate and invertebrate embryonic development, tumor growth, angiogenesis, wound healing, and physiology (2, 5–7).

Each FGFR contains an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. The extracellular ligand binding domain of the FGFR contains two or three Ig-like domains (8, 9). Alternative RNA splicing that utilizes one of two unique exons results in two different versions of Ig-like domain III (referred to as domains IIIb and IIIc) in FGFRs 1–3. This alternative splicing is an essential determinant of ligand binding specificity (10–14). The IIIa ("a") splice form encodes a secreted extracellular FGF-binding protein with no known signaling capability (13). The IIIb ("b") and IIIc ("c") splice forms are regulated in a tissue-specific manner, such that the b isoform is restricted to epithelial lineages and the c isoform is preferentially expressed in mesenchymal lineages (15–18). This tissue specificity is particularly important for FGFR2 function. The structural basis by which this major alternative splicing in Ig-like domain III of FGFR2 modulates FGF binding specificity has been elucidated (14). In addition to tissue-specific FGF and FGFR expression, FGFR activation is modulated by heparin, heparan sulfate, or other glycosaminoglycan chains (19–23). Importantly, tissue-specific alterations in glycosaminoglycan structure provide a mechanism to modulate FGF signaling (24–27). It has been recently postulated that the HS selectivity in FGF signaling is attained in the context of a 2:2 FGF-FGFR dimer and not by FGF, FGFR, or even 1:1 FGF-FGFR pairs (28).

The identification of FGF-FGFR binding specificities is critical to understanding the biological mechanisms involved in normal development and pathogenesis. Here we extend our previous study (12) to semiquantitatively compare the activity of all signaling members of the FGF family on the major splice forms of all FGFRs.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant FGF1, FGF7, FGF8, FGF9, FGF10, FGF16, FGF17, FGF18, and FGF20 were from PeproTech Inc. (Rocky Hill, NJ). Recombinant human FGF12, FGF14, FGF19, FGF21, and FGF23 were expressed in Escherichia coli and purified as previously described (29–31). The source of FGF cDNAs, FGFR expression plasmids, and FGFR-expressing BaF3 cell lines were described previously (12).

BaF3 Cell Culture and Mitogenic Assay—BaF3 cells expressing specific FGFRs were described previously (12). Cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% newborn bovine serum (Sigma), 0.5 ng/ml murine-recombinant interleukin-3 (PeproTech Inc.), 2 mM l-glutamine, penicillin-streptomycin, 50 μM β-mercaptoethanol (BaF3 culture medium), and G418 (600 μg/ml).

For mitogenic assays, FGF-FGFR-expressing BaF3 cells were plated at a density of 30,000 cells/well in a 96-well assay plate in BaF3 assay medium containing varying concentrations of FGF and heparin. FGFs diluted in assay medium were added to each well for a total volume of 200 μl/well. The cells were then incubated for 36–48 h. Mitogenic activity was determined by adding 1 μCi of [3H]thymidine in 50 μl of BaF3 assay medium to each well. The cells were harvested after 4–6 h by filtration through glass fiber paper. The incorporated [3H]thymidine was counted on a Wallac 1420 liquid scintillation counter (PerkinElmer Life Sciences).
Preparation of FGF22-conditioned Medium—A cDNA encoding the full-length mouse FGF22 protein was isolated from mouse brain mRNA by reverse transcription-PCR (forward primer, 5'-H11032-AATT-GCTAGCATGCGCAGCCGCCTCTGGCTG-3'; reverse primer, 5'-H11032-AGGCCTCGAGAGACGAGACCAAGACTGGCAG-3') (32). The PCR product (~500 bp) was inserted into the NheI-XhoI sites of the APtag5 vector (GenHunter Inc.), replacing both the signal sequence and the alkaline phosphatase sequence. APtag5-mFGF22 or the APtag5 empty vector was transfected into baby hamster kidney (BHK) cells (cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin). The cells were replated at 80–90% confluence into 90-mm dishes the day before transfection and then were transfected with 24 μg of plasmid and 60 μl of Lipofectamine 2000 in 1.5 ml of Opti-MEM (Invitrogen) medium according to the manufacturer's directions. After 48 h, the cells were split 1:20 into Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin, and 500 μg/ml Zeocin (Invitrogen). Isolated colonies were picked after 10–14 days and propagated in the same medium. Reverse transcription-PCR (forward primer, 5'-GCTTCTATGTCGAGCACTGGCAGGCATCAGCAGG-CAGCAG-3'; reverse primer, 5'-AGGAGACTGCAGAAGAACATGCGGAGAATGTC-3') was used to identify stable clonal cell lines expressing high levels of APTag5-FGF22 (see Fig. 1A).

Subconfluent stably transfected cell lines, grown in selection medium, were washed twice with phosphate-buffered saline and allowed to grow in BaF3 assay medium containing 2 μg/ml heparin for 48–72 h. Conditioned medium from control APtag5 cells and APTag5-FGF22 cells was collected, centrifuged to remove debris, and used for subsequent BaF3 assays.

Data Analysis—FGF1 was used as a positive control to normalize the mitogenic activity of the other FGFs. To reduce sampling error in the comparison of the relative mitogenic activity, the mitogenic activity for each ligand was averaged at two different concentrations (312 and 1250 pM) (see Table 1) and normalized to the activity of FGF1 at these concentrations, or in the case of FGF19, -21, -23 to 5 nM FGF1.

RESULTS
Receptor Binding Specificity of FGF Subfamilies—The murine pro-B cell line, BaF3, is an interleukin-3-dependent cell line that is commonly used for the analyses of receptor tyrosine kinase activity. Wild-type BaF3 cells do not express FGFRs, and BaF3 cells transfected with FGFRs proliferate in the absence of interleukin-3 when stimulated with FGF and heparin (19). Receptor binding specificity for FGFs 1–9 has been previously described (12). To directly compare the activity of FGF subfamilies containing newly described members, all seven FGFR-expressing BaF3 cell lines were assayed with each FGF at concentrations rang-
FGF family is one of the largest growth factor families, consisting of 22 members sharing 13–71% sequence similarity in mammals (2). FGFs possess a large range of activities in embryonic development and physiological functions in the adult. In the embryo, FGFs often signal across mesenchymal-epithelial boundaries, where FGFR-expressing cells. FGFs 9 and 16 showed no activity toward FGFR1b, 2b, or 3b (Fig. 4, C and D; Fig. 5 and Table 1).

The FGF11 family has no known activity toward any FGF (30, 42, 43). Recombinant FG12 and FG14 protein showed no activity on any of the BaF3 cell lines, consistent with previous observations (30) (data not shown).

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Receptor Specificity of the FGF Family

FIGURE 3. Receptor activation by FGFs 9, 16, and 20. A, BaF3 cell mitogenic assay for c-spliced FGFRs. B, BaF3 cell mitogenic assay for b-spliced FGFRs and FGFR4.A.

they regulate organogenesis and pattern formation (44–47). In the adult, FGFs play important roles in regulating homeostasis, wound healing, and tissue repair (48). Unregulated expression of FGFs can cause cancer (41, 49, 50).

The expression patterns of FGFR receptors 1, 2, and 3 are distinct but overlap in some tissues. Analysis of the alternative splicing pattern of these receptors demonstrates that the utilization of either the b or c exon is dependent upon cell lineage. The b isoform is preferentially expressed in epithelial tissues, whereas the c isoform is expressed in mesenchymal tissues (17, 51–53). The activity of several of the FGFs can be divided along these lines. For example, the FGF7 subfamily is expressed in mesenchyme and shows the greatest activity toward FGFR2b. The FGF8 subfamily is expressed in epithelial tissues and activates c splice forms of FGFRs. Binding specificity between FGF and FGFRs is thus critical for the spatial regulation of FGF signaling.

The specificity of the FGF7 subfamily, demonstrated in vitro in the BaF3 cell assay (Fig. 5), closely reflects the in vivo function of FGF10, a molecule expressed in mesenchymal tissue that signals to epithelial FGFR2b. Disruption of FGF10 signaling is catastrophic to embryonic development, resulting in severe defects in limb development and in organs requiring branching morphogenesis, such as the pancreas, salivary glands, and lungs (54–60). The interaction between FGF10 and FGFR2b is also required for embryonic palate and cecum development (61, 62). Mutations in FGFR2 that alter ligand binding specificity also result in developmental disease. Apert syndrome is a debilitating disease involving craniosynostosis, syndactyly, and mental retardation (63–65). The molecular etiology of Apert syndrome stems from inappropriate activation of FGFR2c by FGF7 family members (31, 66–69).

The crystal structure of FGF10 in complex with FGFR2b has revealed that specific contacts between residues from the alternatively spaced βC′–βE loop region of FGFR2b and residues in the β4 strand and N terminus of FGF10 account for the FGF10-FGFR2b binding specificity (14). In particular, two highly specific hydrogen bonds involving Asp-76 in the N terminus of FGF10 and Ser-315 in the βC′–βE loop of FGFR2b are essential for FGF10-FGFR2b specificity. The involvement of these hydrogen bonds in conferring specificity between the FGF7 subfamily and FGFR2b is further supported by the fact that Asp-76 is unique to the FGF7 subfamily and Ser-315 is located in the b splice isoform-specific βC′–βE loop (14).

FGF8 subfamily members exclusively bind FGFRc splice forms and FGFR4 (Fig. 5). The recent crystal structure of FGF8b in complex with FGFR2c has provided the molecular basis by which FGF8 subfamily members attain their specificity/promiscuity toward c splice isoforms of FGFRs 1–3 and FGFR4 (70). Importantly, in the case of the FGF8 subfamily, the alternatively spliced βE and βG strands, and not the βC′–βE loop, harbor the primary receptor determinants of FGF8 subfamily binding specificity. This is because the spatial positioning of the N terminus of FGF8 subfamily members, relative to the β-trefoil core, is opposite to that of other FGFs (70).

FGFs 8, 17, and 18 have distinct and overlapping expression patterns in several tissues, such as the developing mid-hindbrain junction (71–73). Several of these FGFs are required for central nervous system morphogenesis (33, 70, 74–76). Fgf8 and Fgf17 are expressed in the apical ectodermal ridge during limb development, and inactivation of Fgf8 in early limb ectoderm causes a reduction in limb-bud size, demonstrating that FGF8 signaling from the apical ectodermal ridge is essential for normal limb development (77). Fgf18 is expressed in the perichordium, where it regulates chondrogenesis and osteogenesis (78, 79).

The FGF9 subfamily activates all FGFRc isoforms and FGFR3b (Fig. 5). FGF9 is widely expressed in epithelial-like and neuronal tissues during embryonic development and is required for lung, heart, inner ear, digestive system, and testes development (35, 80–88). In the BaF3 mitogenic assay, FGF19 subfamily members have weak activity compared with FGF1. However, at high concentrations, these FGFs activate FGFR1c, -2c, -3c, and FGFR4 (Fig. 5). Compared with other FGF subfamilies that have high affinities for FGFRs and regulate cell proliferation, differentiation, and migration, these subfamily members appear to serve as metabolic regulators and hormones (36, 37, 40, 89–94). It is possible that other cofactors are required for optimal activity or that these FGFs interact with and activate other types of receptors. Recently,
the Klotho protein was found to serve as a coreceptor for FGF23 binding
to FGFR1c, -3c, and -4, and in signaling assays, Klotho enhanced FGF23
activity over 10-fold (95). Additionally, kidney epithelial cells may
express an FGF23 receptor on their basolateral surface that is function-
ally distinct from known FGFRs (96).

As previously reported, FGFs 12 and 14 showed no mitogenic activity
in the BaF3 assay (Fig. 5) (30). The FGF11–14 subfamily, also termed
FGF homologous factors (FHFs), contain sequence homology to the
core region of FGFs (97–100). Indeed, the crystal structure of FHF1b
(FGF12b) shows that the core region of FHFs superimpose very well
onto that of bona fide FGFs such as FGF9 (30). The structure shows that
two FHF-invariant surface residues in the core region of FHFs, Arg-52
and Val-95 in the FHF1b, contribute to the inability of FHFs to bind
FGFRs (30). FHFs differ from most other FGFs in that they lack an N-terminal signal sequence. FHFs are
expressed at highest levels in the developing and mature nervous system
(100, 101) where they are important for neurophysiological function
(102–104). Interestingly, members of the FHF family have been found to
interact with cytoplasmic proteins, such as IB2, and the C-terminal tail
of voltage-gated sodium channels (42, 43, 105–109).

FGF1 is the universal FGF and can activate all FGFRs. Comparison of
the crystal structures of FGF1-FGFR1c, FGF1-FGFR2c, and FGF1-
FGFR3c complexes has provided key insights into the unique FGFR
binding promiscuity of FGF1 (110). FGF1 was used as an internal con-
trol to normalize the relative activity of other FGFs. The diversity of FGF
receptor binding specificity is summarized in Table 1 and Fig. 5.
relative activity of these FGFs can be affected by the quality of the recombinant protein and may account for quantitative differences between the data shown here and that described previously using the same assay (12). A quantitative systematic analysis of all possible FGF-FGFR binding interactions is being carried out by Mohammadi et al. (4) using surface plasmon resonance (see Table 1 in Ref. 4). Overall, the currently available surface plasmon resonance data are in agreement with the results of the mitogenesis-based analysis presented here. The few differences between these data sets may be due to the fact that the surface plasmon resonance experiments are carried out in the absence of heparin/HS to solely monitor 1:1 FGF-FGFR binding, whereas the mitogenic assays are in the presence of exogenous heparin.

The in vivo specificity of FGFs may also diverge from these in vitro data as, in vivo, many cofactors may modulate FGF affinity for FGFRs. Additionally, the possibility of heterodimer formation between FGFs and FGFRs may further increase the repertoire of receptor-ligand interactions. With these caveats in mind, the data shown here, assayed under the same assay (12). A quantitative systematic analysis of all possible FGF-recombinant protein and may account for quantitative differences with the results of the mitogenesis-based analysis presented here. The overall, the data as, 

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