Receptor Specificity of the Fibroblast Growth Factor Family:

THE COMPLETE MAMMALIAN FGF FAMILY*

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Abstract

In mammals, 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.

Fibroblast growth factors (FGFs)[2] 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 2:2:2 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

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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 semi-quantitatively 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 FGFR 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 (Pepro-Tech Inc.), 2 mM L-glutamine, penicillin-streptomycin, 50 nM β-mercaptoethanol (BaF3 culture medium), and G418 (600 μg/ml).

For mitogenic assays, 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 [^{3}H]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 [^{3}H]thymidine was counted on a Wallac β plate 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′-AATTGCTAGCATGCGCAGCCGCCTCTGGCTG-3′; reverse primer, 5′-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, 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′- GCTTCTATGTGGCCATGAATCGCA-3′; reverse primer, 5′- AGACCAAGACTGGCAGGAAGTGT-3′) was used to identify stable clonal cell lines expressing high levels of Aptag5-FGF22 (see Fig. 1*A*).

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 APtag-5 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 ranging from 20 to 5000 pM (FGFs 1, 7–10, 16–18, and 20) and from 3 to 800 nM (FGFs 19, 21, and 23).

FGFs 7, 10, and 22 are more closely related to each other by sequence than to any other FGF and were predicted to have similar activity toward FGFRs. We and others have failed to produce active recombinant FGF22 protein. We therefore developed a cell line that secretes FGF22 into the culture medium (Fig. 1*A*). Dilutions of conditioned medium were assayed on FGFR2b- BaF3 cells. The concentration of the FGF22-conditioned medium was estimated to be equivalent to an FGF10 concentration of 300–600 pM. Control-conditioned medium showed no activity toward any of the BaF3 cell lines. FGFs 7, 10, and 22 all strongly activated FGFR2b. FGFs 10 and 22 showed weak activity toward FGFR1b, and all three of these FGFs failed to activate any of the other FGFR-expressing cell lines (Fig. 1, *B* and *C*; Fig. 5; and Table 1).

The FGFs 8, 17, and 18 subfamily members showed similar activities to each other as those previously described (33,34). These FGFs specifically activated FGFRs 1c, 2c, and 3c and the two Ig-like domain forms of FGFR4 (4Δ). FGF8 subfamily members showed higher relative activity on FGFR3c cells and less activity toward FGFR1c. No activity was observed on the FGFRb isoform-expressing cell lines (Figs. 2 and 5 and Table 1).

The FGFs 9, 16, and 20 subfamily members showed a similar profile to that of FGF9 (12,35). These FGFs activated FGFRc isoforms and FGFR4 with high activity, and unlike other FGFs that preferentially activate c splice form receptors, these FGFs consistently showed activity

toward FGFR3b-expressing cells. FGF20 showed slight activity on FGFR2b cells and no activity on FGFR1b cells. FGFs 9 and 16 showed no activity toward FGFR1b or -2b (Figs. 3 and 5 and Table 1).

The FGF19 (mouse FGF15), FGF21, and FGF23 subfamily is unique in that these FGFs can behave as secreted hormones (36–41). To obtain significant activity of these FGFs on FGFR-expressing BaF3 cells required a protein concentration ranging from 3 to 800 nM and a heparin concentration >10 μg/ml (Fig. 4*A*). These parameters indicated low relative activity compared with other classic members of the FGF family. However, at these concentrations, FGFs 19, 21, and 23 showed consistent activity toward FGFR1c, 2c, 3c, and FGFR4 but 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 FGFR (30,42,43). Recombinant FGF12 and FGF14 protein showed no activity on any of the FGFR-expressing BaF3 cell lines, consistent with previous observations (30) (data not shown).

DISCUSSION

The 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 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 FGF 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 mesenchymal 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 spliced 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 $\beta C'$–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 βF and βG strands, and not the $\beta C'$–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 perichondrium, 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 mito-genic 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 functionally 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 in the $\beta 4$–$\beta 5$ loop and Val-95 in the $\beta 9$ strand of 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 control to normalize the relative activity of other FGFs. The diversity of FGF receptor binding specificity is summarized in Table 1 and Fig. 5. The 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 identical experimental conditions, should provide a base line for comparison of relative FGF activity and specificity and should allow predictions of potential signaling between FGF ligands and FGF receptors in development, in tissue homeostasis, and in disease.

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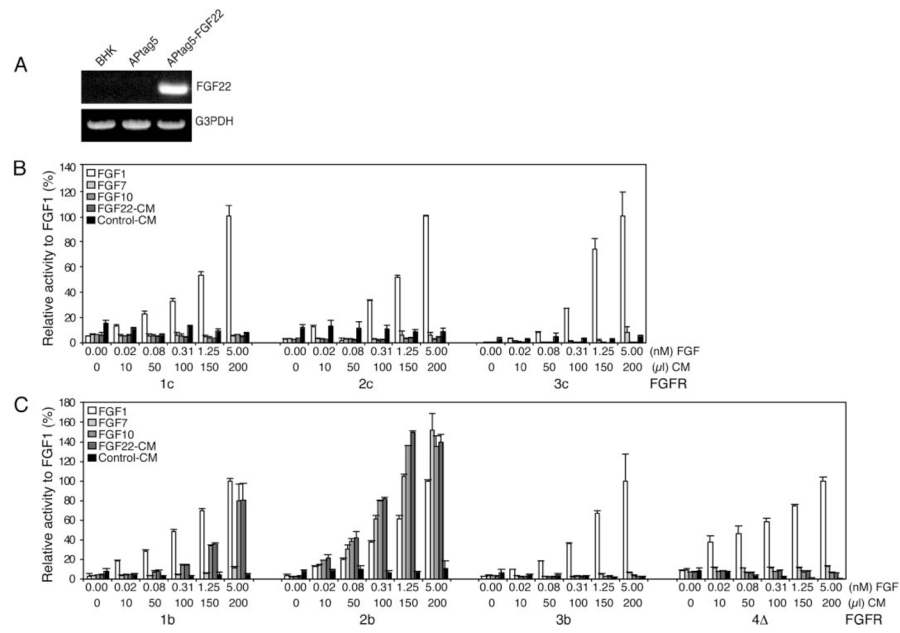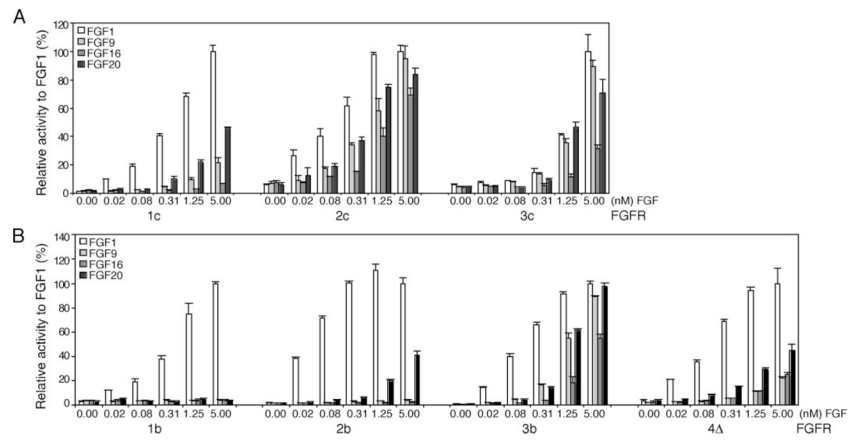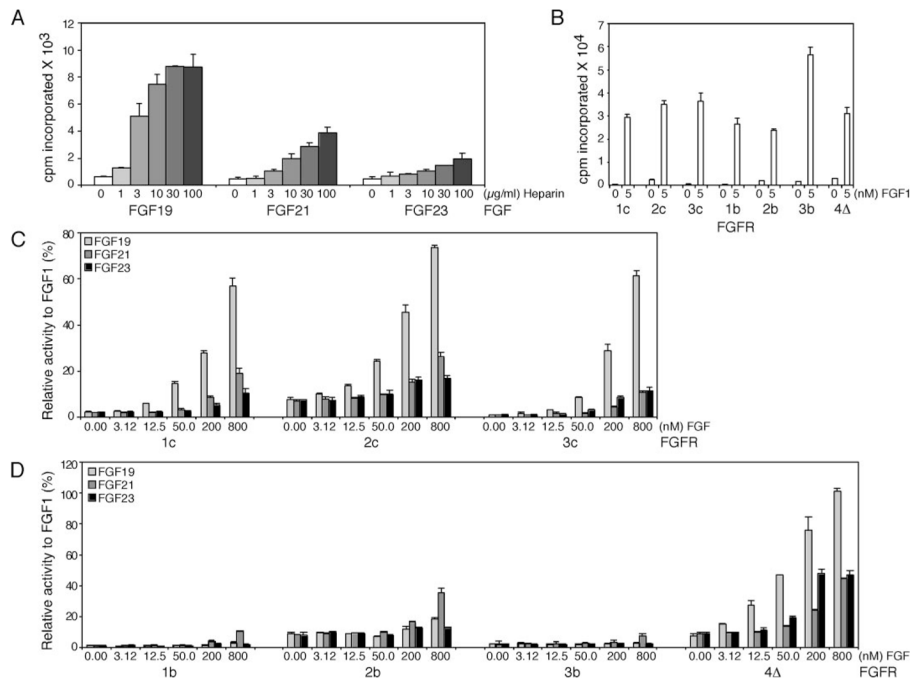
FIGURE 1. Receptor activation by FGFs 7, 10, and 22
A, FGF22 expression in BHK cells stably transfected with expression plasmid APtag5-FGF22 (BHK/ATtag5-FGF22) or empty vector APtag5 (BHK/APtag5). Individual Zeocin-resistant clones were selected and screened by reverse transcription-PCR for Fgf22 expression. *Lane 1*, wild-type BHK cells do not express FGF22; *lane 2*, BHK cells transfected with the APtag5 plasmid do not express FGF22; *lane 3*, BHK cells transfected with APtag5-FGF22 express high levels of FGF22. *B*, BaF3 cell mitogenic assay for c-spliced FGFRs. *C*, BaF3 cell mitogenic assay for b-spliced FGFRs and FGFR4Δ. *CM*, conditioned medium.
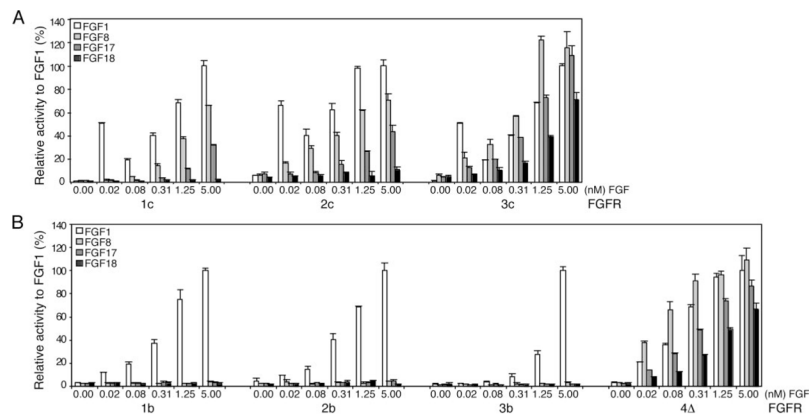
FIGURE 2. Receptor activation by FGFs 8, -17, and -18
A, BaF3 cell mitogenic assay for c-spliced FGFRs. *B*, BaF3 cell mitogenic assay for b-spliced FGFRs and FGFR4Δ.

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 Δ.

FIGURE 4. Receptor activation by FGFs 19, 21, and 23
A, heparin dose response for FGFs 19, 21, and 23 measured on FGFR1c-expressing BaF3 cells. Activity increases with increasing heparin but appears to plateau at concentrations >10 μg/ml. *B*, FGF1 control activity used to normalize the activity of FGFs 19, 21, and 23. *C*, BaF3 cell mitogenic assay for c-spliced FGFRs. *D*, BaF3 cell mitogenic assay for b-spliced FGFRs and FGFR4Δ.
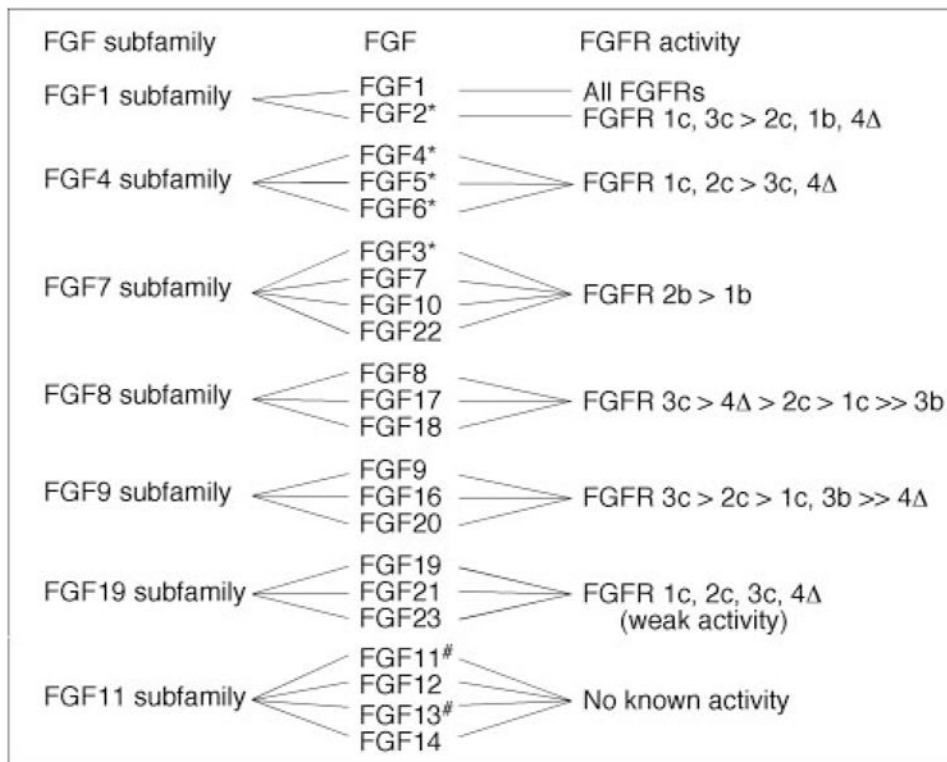
FIGURE 5. Diagram showing relative activity of FGFs grouped by subfamilies
*, data from Ornitz *et al.* (12); #, not tested.

TABLE 1

Relative mitogenic activity of FGF subfamilies

FGF receptor	Relative mitogenic activity ± S.D.												
	FGF-1	FGF7 subfamily			FGF8 subfamily			FGF9 subfamily			FGF19 subfamily (×10³)		
	FGF-1	FGF7	FGF10	FGF22	FGF8	FGF17	FGF18	FGF9	FGF16	FGF20	FGF19	FGF21	FGF23
1c	100 ± 5	14.2 ± 4.1	12.5 ± 2.5	10.2 ± 1.7	57.5 ± 3.2	22.7 ± 0.3	4.7 ± 0.4	12.5 ± 1.4	4.3 ± 0.3	28.1 ± 4.0	123 ± 5.7	34 ± 3.0	23 ± 2.7
2c	100 ± 3	10.4 ± 4.0	6.1 ± 1.7	7.1 ± 1.1	91.6 ± 8.9	27.1 ± 9.5	28.9 ± 4.7	57.2 ± 5.4	32.5 ± 3.5	68.4 ± 2.8	238 ± 11.5	86 ± 4.7	89 ± 10.5
3c	100 ± 6	3.3 ± 1.4	0.8 ± 0.9	1.0 ± 0.5	209 ± 6	111 ± 5	77.7 ± 3.4	90.4 ± 6.0	32.4 ± 6.8	89.5 ± 7.3	140 ± 11.1	23 ± 02.3	43 ± 5.5
1b	100 ± 4	8.0 ± 1.5	39.4 ± 1.3	40.3 ± 1.6	5.3 ± 0.4	6.0 ± 2.2	6.3 ± 1.1	7.3 ± 1.0	6.5 ± 1.1	7.3 ± 0.9	12 ± 0.5	19 ± 3.4	11 ± 2.4
2b	100 ± 5	168 ± 6	217 ± 1	232 ± 3	5.9 ± 1.4	6.3 ± 0.6	7.8 ± 1.8	2.9 ± 0.4	1.8 ± 0.5	12.3 ± 0.7	61 ± 6.9	83 ± 5.4	66 ± 2.7
3b	100 ± 4	6.7 ± 1.8	6.0 ± 0.5	5.3 ± 0.7	18.6 ± 6.3	10.7 ± 2.7	12.5 ± 2.0	42.7 ± 2.9	13 ± 2.6	44.3 ± 1.5	20 ± 1.7	26 ± 1.5	21 ± 0.8
4Δ	100 ± 4	17.8 ± 0.5	11.5 ± 0.5	12.5 ± 0.9	102 ± 0.4	85.5 ± 1.1	52.8 ± 1.7	10.1 ± 0.1	9.9 ± 0.3	26.6 ± 0.9	410 ± 27.7	128 ± 9.6	222 ± 11.9