# Fibroblast Growth Factor Receptors Have Different Signaling and Mitogenic Potentials

JEN-KUEI WANG,[1,2] GUANGXIA GAO,[1,2] AND MITCHELL GOLDFARB[1]*

*Regeneron Pharmaceuticals, Inc., Tarrytown, New York 10591,[1] and Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, New York 10032[2]*

Fibroblast growth factor (FGF) receptors (FGFRs) are structurally related receptor protein tyrosine kinases encoded by four distinct genes. Activation of FGFR-1, -2, and -3 by FGFs induces mitogenic responses in various cell types, but the mitogenic potential of FGFR-4 has not been previously explored. We have compared the properties of BaF3 murine lymphoid cells and L6 rat myoblast cells engineered to express FGFR-1 or FGFR-4. Acidic FGF binds with high affinity to and elicits tyrosine phosphorylation of FGFR-1 or FGFR-4 receptors displayed on BaF3 cells, but only FGFR-1 activation leads to cell survival and growth. FGFR-4 activation also fails to elicit detectable signals characteristic of the FGFR-1 response: tyrosine phosphorylation of SHC and extracellular signal-related kinase (ERK) proteins and induction of *fos* and *tis11* RNA expression. The only detected response to FGFR-4 activation was weak phosphorylation of phospholipase Cγ. A chimeric receptor containing the extracellular domain of FGFR-4 and the intracellular domain of FGFR-1 confers FGF-dependent growth upon transfected BaF3 cells, demonstrating that the intracellular domains of the receptors dictate their functional capacity. Activation of FGFR-1 in transfected L6 myoblasts induced far stronger phosphorylation of phospholipase Cγ, SHC, and ERK proteins than could activation of FGFR-4 in L6 cells, and only FGFR-1 activation induced tyrosine phosphorylation of a characteristic 80-kD protein. Hence, the signaling and biological responses elicited by different FGF receptors substantially differ.

Fibroblast growth factors (FGFs) comprise a family of polypeptide ligands which mediate biological responses in many kinds of differentiated cells (reviewed in reference 2). FGFs can serve as chemoattractants for vascular endothelial cells and as survival factors for postmitotic neurons. Furthermore, FGFs can act as promoters or inhibitors of cellular differentiation. Lastly, FGFs stimulate proliferation of a wide range of ectoderm- and mesoderm-derived cell types.

FGF responses are triggered by the activation of FGF receptors (FGFRs), which are a family of structurally related transmembrane tyrosine kinases. FGFR activation elicits tyrosine phosphorylation of the receptor itself and of intracellular proteins, including phospholipase Cγ (PLCγ) (1), of extracellular signal-regulated kinases (ERKs) (5), and of one or several uncharacterized proteins of 80 to 90 kDa (6, 11, 23). Differences in ectodomain sequence confer upon each FGFR a distinct profile of affinities towards the different FGFs (7, 20, 25, 27, 39, 42). More subtle differences in cytoplasmic domain sequence might be expected to confer different signaling and biological potentials upon FGFRs, and this contention is supported by the existence of a bladder tumor cell line which undergoes different physiological changes in response to different FGFs (38). However, most studies to date have highlighted similarities in FGFR-mediated responses. For example, activation of FGFR-1 or FGFR-3 ectopically expressed in *Xenopus* oocytes triggers a calcium influx response (12, 13), and activation of FGFR-1, -2, or -3 expressed in transfected murine BaF3 pro-B-lymphoid cells triggers cell proliferation (3a, 25). The only documented difference in signaling potential among FGFRs is the ability of FGFR-1, but not FGFR-4, to stimulate

phosphorylation of PLCγ in transfected Chinese hamster ovary (CHO) cells (36).

Here we report our characterization of the responses elicited by FGFR-1 versus FGFR-4 in transfected BaF3 and rat L6 myoblast cells. In contrast to FGFR-1, activation of FGFR-4 in BaF3 cells has a minimal effect on tyrosine phosphorylation of intracellular signaling molecules, fails to induce expression of *fos* and *tis11* RNAs, and does not promote a mitogenic response. Activation of a chimeric receptor bearing the FGFR-4 ectodomain and FGFR-1 cytoplasmic domain stimulates BaF3 cell proliferation, demonstrating that the intracellular domains of these receptors are responsible for their different functional potentials. We also find that activation of FGFR-1 triggers a broader and stronger protein tyrosine phosphorylation response in transfected L6 cells than does activation of FGFR-4.

## MATERIALS AND METHODS

**Reagents.** Human recombinant acidic FGF and FGF-4 were generously provided by X. Zhan and C. Basilico, respectively. Acidic FGF was also purchased from R&D Systems, along with human recombinant basic FGF. Human recombinant FGF-5 was purified as described previously (4). Human recombinant keratinocyte growth factor (KGF) was purchased from Pepro Tech Inc. Rabbit polyclonal FGFR-4 antisera were raised against the peptide CGGSPFPFSD-SQTT, the C-terminal 11 amino acids of murine FGFR-4 tagged with CGG for conjugation to carrier. Rabbit polyclonal anti-FGFR-1 immunoglobulin G (IgG) was obtained from Santa Cruz Biotechnology, Inc., mouse antiphosphotyrosine monoclonal antibody 4G10, anti-bovine PLCγ-1 antibody, and anti-human SHC polyclonal antibody were purchased from Upstate Biotechnology, Inc., rabbit anti-ERK antibodies were generously provided by T. Boulton, and horseradish peroxidase-conjugated anti-mouse and anti-

* Corresponding author. Mailing address: Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707. Phone: (914) 345-7455. Fax: (914) 345-7650.

rabbit Ig antibodies were from Promega and Sigma Chemical, respectively. Prestained protein molecular weight standards were purchased from GIBCO/BRL.

**FGFR cloning and expression constructs.** A segment of murine FGFR-4 cDNA was cloned following PCR amplification using FGFR-specific oligonucleotide primers and a murine embryoid body cDNA library template as previously described (17, 43). This fragment was used as a hybridization probe to isolate murine FGFR-4.1 and FGFR-4.2 cDNA clones from the mouse embryonic day 6.5 and 7.5 cDNA libraries, respectively. These libraries were kindly provided by J. Gerhart. A similarly derived PCR segment of murine FGFR-1 cDNA was used as probe to isolate from the embryonic day 6.5 library a full-length cDNA encoding the short two-Ig-domain form of murine FGFR-1 lacking the outer Ig domain (12). These cDNA clones were inserted as EcoRI fragments into the mammalian expression vector pvcos (44) to generate pvcosFR4.1, pvcosFR4.2, and pvcosFR1S. pMoFR1, an expression vector bearing the long three-Ig-domain form of murine FGFR-1 cDNA (41), was kindly provided by D. Ornitz.

The FGFR-4/FGFR-1 chimera (FR4/R1C) was constructed so as to contain the extracellular domain of FGFR-4 and the transmembrane and intracytoplasmic domains of FGFR-1, taking advantage of an EcoRV cleavage site located precisely at the ectodomain-transmembrane border of FGFR-4 cDNA. The chimera was constructed in pBluescriptII KS vector (Stratagene) by ligation of three fragments: (i) a SalI-partial EcoRV 4.5-kbp fragment containing the FGFR-4 ectodomain linked to pBluescriptII KS, (ii) a BamHI-SalI 1.8-kbp fragment encoding most of the murine FGFR-1 intracytoplasmic domain and 3′ untranslated sequence, and (iii) a 280-bp EcoRV-BamHI FGFR-1 PCR fragment extending 5′ from that native BamHI site through the transmembrane domain, using PCR primer-induced silent mutation to generate the EcoRV site. The chimeric cDNA was then excised with EcoRI and inserted into pvcos.

**Transfection of BaF3 and L6 cells.** BaF3 mouse pro-B cells (19, 26) were cultured in RPMI 1640 (GIBCO/BRL) supplemented with 10% fetal calf serum (GIBCO), 2 μg of L-glutamine per ml, and 20% WEHI-3 cell conditioned medium as a source of interleukin-3 (IL-3). BaF3 cells ($2 \times 10^7$ in 0.5 ml of phosphate-buffered saline [PBS]) were transfected with 20 μg of linearized plasmid by electroporation, using a Bio-Rad Gene Pulser set at 200 V and 960 μF. For some transfections, 10 μg each of pvcosFGFR and pLTRneo (44) was linearized with MluI and ligated into concatemers prior to electroporation. Electroporated cells were allowed to grow for 2 days in complete medium (containing IL-3) for stable transfectants to establish and then plated into microwells for direct FGFR selection with acidic FGF (10 ng/ml) plus heparin (10 μg/ml; Sigma) in the absence of IL-3, or for indirect selection with G418 (400 μg/ml; GIBCO) plus IL-3 to generate G418-resistant clones, which were subsequently assayed for FGFR expression by ability to bind ^{125}I-labeled acidic FGF (see below).

The rat L6 myoblasts were obtained from the American Type Culture Collection and cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum. Cells were cotransfected with plasmids and carrier DNA by the calcium phosphate method (40), using 1 μg of pLTRneo and 2 μg of FGFR expression vector DNA per 100-mm-diameter dish. Clones were isolated after 12 days of selection in 800 μg of G418 per ml and screened for expression of FGFR by ability to bind radiolabeled FGF.

**BaF3/FGFR cell proliferation assay.** BaF3 cell lines were washed once with RPMI 1640 and plated at $10^4$ cells per well in 96-well culture dishes. Growth factors and heparin (10 μg/ml) were added in a total volume of 0.2 ml of RPMI 1640–10% fetal calf serum without IL-3. After 3 days of growth, relative viable cell numbers were monitored by the dimethylthiazolyl-diphenyltetrazolium bromide (MTT) assay (Promega).

**FGF/FGFR binding, competition, and cross-linking assays.** Acidic FGF was labeled with ^{125}I, using the protocol for Enzymobeads (Bio-Rad), and protein labeled to specific activity of ~2 × $10^5$ cpm/ng was purified by heparin-Sepharose batch binding and 2 M NaCl elution. BaF3 cells expressing FGFRs were washed once with RPMI 1640, and $2 \times 10^5$-cell aliquots were each resuspended in 0.25 ml of cold binding buffer (RPMI 1640, 1 mg of bovine serum albumin [BSA] per ml, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.5], 50 μg of heparin per ml) plus iodinated FGF at varied concentration and incubated for 7 h at 4°C with gentle shaking. The cells were washed twice with ice-cold PBS-heparin, lysed in 0.3 M NaOH, and counted by gamma scintillation. Nonspecific binding was determined by parallel binding experiments with parental BaF3 cells. Binding data were plotted and used to determine $K_d$ by an established procedure (31). For competition assays, aliquots of $10^6$ BaF3/FGFR cells were incubated in cold binding buffer containing 150 pM ^{125}I-labeled acidic FGF plus competing unlabeled FGFs at varied concentration and then incubated and processed as described above. For cross-linking studies, $10^6$ cells were washed and incubated as described above with 250 pM ^{125}I-labeled acidic FGF in the presence or absence of unlabeled acidic or basic FGF at 50-fold excess. After three washes with ice-cold PBS–1 mg of BSA per ml–50 μg of heparin per ml, cells were resuspended and incubated in 250 μl of cross-linking solution (PBS, 0.2 mM disuccinimidyl suberate [Pierce Chemical], 1 mg of BSA per ml, 50 μg of heparin per ml, 1 mg of glucose per ml) for 45 min at room temperature, then quenched with 500 μl of TN buffer (50 mM Tris [pH 7.4], 150 mM NaCl), washed twice with TN, and solubilized at 0°C in radioimmunoprecipitation assay buffer (PBS, 0.5% deoxycholic acid, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride–10 μg of aprotinin per ml). After 10 min on ice, the nuclei were removed by centrifugation and the supernatants were analyzed by electrophoresis through a sodium dodecyl sulfate (SDS)–6% polyacrylamide gel and autoradiography.

To detect and quantitate FGFRs on transfected L6 clones, $10^5$ cells plated on a gelatinized 16-mm-diameter culture well were incubated on ice with 0.25 ml of binding buffer containing 1 nM ^{125}I-labeled acidic FGF with or without a 50-fold excess cold acidic FGF for 4 h and then washed three times on ice with binding buffer, and bound radiolabel was quantitated after solubilization. Specific (competable) binding together with the specific activity of the labeled FGF allowed determination of FGFR density.

**Protein tyrosine phosphorylation in BaF3 cells.** Cells were washed and then starved for 16 h in medium lacking IL-3. After exposure to acidic FGF (200 ng/ml) plus heparin (25 μg/ml) at 37°C for 10 min, cells were washed once with cold lysis buffer without detergent (20 mM Tris [pH 7.6], 150 mM NaCl, 50 mM NaF, 1 mM $Na_3VO_4$, 5 mM benzamidine, 1 mM EDTA, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride) and resuspended in lysis buffer with 1% Nonidet P-40. To detect tyrosine phosphorylation of FGFRs, PLCγ, and SHC, clarified lysates (1 mg) were immunoprecipitated with antibodies to the respective proteins, and the immune complexes were recovered by

using protein G-Sepharose (Pharmacia LKB), solubilized in SDS sample buffer (16), and electrophoresed through SDS–7.5% polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and assayed for phosphotyrosine as described elsewhere (33), using antiphosphotyrosine antibodies and enhanced chemiluminescence reagents (Amersham) for development. The immunoblots of the PLCγ and SHC immunoprecipitates were stripped of antibodies with 62 mM Tris (pH 6.7)–2% SDS–0.1 M β-mercaptoethanol at 50°C for 30 min and reprobed with anti-PLCγ or anti-SHC to confirm the migration and abundance of these proteins in the immunoprecipitates. Tyrosine phosphorylation of ERKs was detected by immunoprecipitation of lysates (1 mg) with antiphosphotyrosine-agarose conjugate, electrophoresis, transfer to polyvinylidene difluoride membranes, and immunoblotting with anti-ERK antibody. Total lysates (60 μg) without immunoprecipitation were included on the gels to monitor ERK migration and abundance.

*fos* and *tis11* gene expression in BaF3/FGFR cells. Quiescent BaF3 and BaF3/FGFR cells were stimulated with acidic FGF (200 ng/ml) plus heparin (25 μg/ml) for 5 to 120 min at 37°C, and RNA was prepared by guanidium thiocyanate lysis and organic extraction (3). Ten micrograms of total RNA was electrophoresed on 1% agarose formaldehyde gels, transferred to nylon membranes, and hybridized to a $^{32}$P-labeled v-*fos* or $^{32}$P-labeled *tis11* probe as described elsewhere (32).

Phosphoprotein analysis in L6 cells expressing FGFRs. L6 myoblast-derived cell lines were starved for 2 h in serum-free Dulbecco modified Eagle medium, left unstimulated or challenged with acidic FGF (100 ng/ml) plus heparin (10 μg/ml) for 5 min and then rinsed and lysed in cold lysis buffer with 1% Nonidet P-40. Aliquots of lysates (250 μg) were immunoprecipitated with various antibodies, and immune complexes were collected by using protein G-Sepharose. Immunoprecipitates and total lysates (50 μg) were electrophoresed, transferred to polyvinylidene difluoride membranes, and assayed for protein phosphotyrosine. For the ERK gel shift experiment, L6-derived cell lines were starved as described above and challenged with acidic FGF (100 ng/ml) plus heparin (10 μg/ml) for 0, 1, or 2 min. Total lysates (50 μg) were electrophoresed, and the immunoblot was probed with anti-ERK antibodies.

## RESULTS

FGFR-4 expression in BaF3 cells does not promote FGF-dependent growth. As part of an effort to characterize the affinities of various FGFs for different FGFRs, we have isolated murine cDNA clones encoding FGFR-4. Two independent clones, FR4.1 and FR4.2, contain the complete FGFR-4 coding sequence, and the longer clone, FR4.2, contains 150 bp of 5' untranslated sequence, thereby extending the previously reported sequence for murine FGFR-4 cDNA (34). Both cDNAs were cloned into expression vector pvcos (44) and transfected into BaF3 cells. Surprisingly, neither FGFR-4 vector could generate transfectants capable of growth with acidic FGF in place of IL-3 (Table 1). By contrast, two different murine FGFR-1 expression vectors generated an abundance of acidic FGF-dependent clones upon transfection into BaF3 cells (Table 1).

To test whether FGFR-4 activation in BaF3 cells causes a weak proliferative response not detected in the colony-forming assay, FGFR-4 vectors were ligated to a G418 resistance vector, and transfected BaF3 cells were selected

TABLE 1. Transformation of BaF3 cells with FGFR expression vectors[a]

| Plasmid | No. of wells containing FGF-dependent clones/ total no. seeded with: | | |
|---|---|---|---|
| | $10^4$ cells | $5 \times 10^4$ cells | $3 \times 10^5$ cells |
| Expt 1 | | | |
| pMoFR1 | 9/48 | 48/48 | 24/24 |
| pvcosFR1S | 16/48 | 48/48 | 24/24 |
| pvcosFR4.1 | 0/48 | 0/48 | 0/24 |
| Expt 2 | | | |
| pvcosFR1S | 1/48 | 11/48 | 16/24 |
| pvcosFR4.2 | 0/48 | 0/48 | 0/24 |
| pvcosFR4/R1C | 0/48 | 11/48 | 11/12 |

[a] In each experiment, 2 × 10⁷ BaF3 cells were electroporated with 20 μg of FGFR cDNA vector, grown for 2 days in complete medium (with IL-3), then plated into microwells containing selection medium (with acidic FGF and heparin, without IL-3) at either 10⁴, 5 × 10⁴, or 3 × 10⁵ cells per well, and cultured for 14 days to allow FGF-dependent clones to emerge.

in IL-3–G418. Some of the resulting G418-resistant clones were found to express FGFR-4 RNA (data not shown) and protein capable of binding radiolabeled acidic FGF (see below). Whereas FGFR-1-expressing BaF3 cells grew when stimulated with either acidic or basic FGF, FGFR-4-expressing cells showed no growth at any tested FGF concentration (Fig. 1), and these cells lost viability to the same extent in the presence or absence of FGF, as reflected both morphologically and in the final MTT uptake values (Fig. 1).

FGFRs on BaF3 cells have normal FGF-binding profiles. The unexpected failure of FGFR-4 vector-transfected BaF3 cells to proliferate in response to FGFs prompted us to test whether transfected BaF3 cells display functional FGFRs. Surface receptors on clones of transfected cells were detected by their ability to bind $^{125}$I-labeled acidic FGF (data not shown). Several clones of FGFR-1- and FGFR-4-transfected BaF3 cells were chosen for extensive binding studies. As shown in Fig. 2A, acidic FGF bound to receptors on FGFR-1-transfected cells with a $K_d$ of 150 pM and to receptors on FGFR-4-transfected cells with a $K_d$ of 600 pM. These affinities are comparable to those reported for acidic FGF toward FGFR-1 and FGFR-4 receptors displayed on adherent cells (10, 27).

The ability of various unlabeled FGFs to compete with labeled acidic FGF for binding to FGFRs on BaF3 cells was used to assess the relative affinities of various FGFs toward FGFR-1 and FGFR-4. Basic FGF bound to FGFR-1 as efficiently as acidic FGF, while FGF-4 and FGF-5 were 10- to 20-fold less avid (Fig. 2B). Acidic FGF bound to FGFR-4 100-fold more efficiently than either FGF-4 or basic FGF and 1,000-fold more efficiently than FGF-5 (Fig. 2C). Neither receptor bound KGF detectably. These relative affinities of FGFs toward FGFR-1 and FGFR-4 were again in general agreement with previous findings in other cell systems (4, 10, 18, 27).

FGF receptors on transfected cells were visualized by chemical cross-linking to radiolabeled acidic FGF followed by SDS-polyacrylamide gel electrophoresis (PAGE). Whereas parental BaF3 cells lacked detectable receptors (Fig. 2D), both FGFR-1 and FGFR-4 BaF3 clones contained receptors which could be cross-linked to labeled acidic FGF. The coupling of labeled acidic FGF to FGFR-1 could be blocked by a 50-fold excess of unlabeled acidic or basic FGF, while labeled acidic FGF/FGFR-4 complex formation
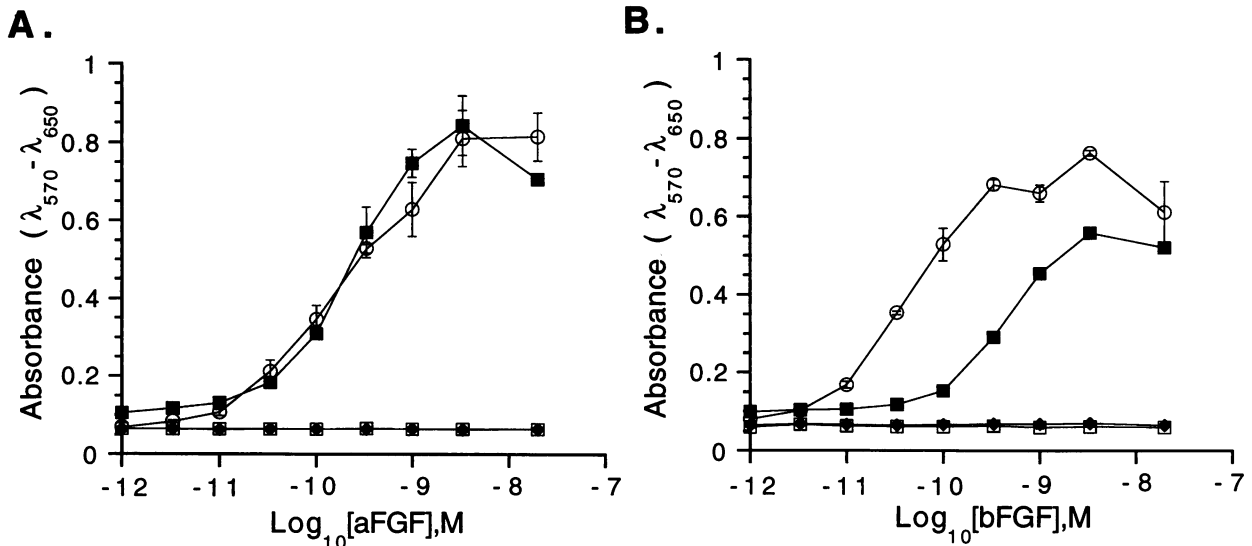
**A.**



**B.**



FIG. 1. FGF-induced proliferation of BaF3/FGFR clones. BaF3/FGFR and parental BaF3 cells were plated in 7-mm-diameter culture wells ($10^4$ cells per well) in the absence of IL-3 and the presence of heparin (10 μg/ml) and different concentrations of acidic FGF (aFGF) (A) or basic FGF (bFGF) (B). Cells were cultured for 3 days, and viable cells were quantitated by MTT uptake, solubilization, and measurement of $A_{570}$ minus $A_{650}$. All values represent the averages of duplicate wells. ○, BaF3/FGFR1-C4; ◆, BaF3/FGFR4-19; ■, BaF3/FGFR4/R1C; □, BaF3.

was blocked only by cold acidic FGF. Although FGFR-1 and FGFR-4 polypeptide chains are of nearly identical length, the apparent molecular weight of FGFR-1 was somewhat larger than that of FGFR-4, as has been observed in another cell system (36).

Hence, by a variety of criteria, FGFRs expressed on BaF3 cells behave indistinguishably from these receptors expressed on other types of cells.

**FGFR-4 activation induces limited signaling in BaF3 cells.** The difference in mitogenic potential between FGFR-1 and FGFR-4 could be due to a difference in the ability of these receptors to become activated after binding ligand. Alternatively, activated FGFRs could have different signaling potentials. We first monitored the levels of FGFR-1 and FGFR-4 tyrosine phosphorylation following ligand induction. BaF3/FR1-C3 and BaF3/FR4-19 cells, which have equivalent numbers of FGFR-1 and FGFR-4 surface receptors, were incubated for 10 min with or without acidic FGF, and cell lysates were immunoprecipitated with antisera raised against C-terminal peptides of either FGFR-1 or FGFR-4. Immunoprecipitates were electrophoresed and Western blotted (immunoblotted) with antiphosphotyrosine antibody. As shown in Fig. 3A, acidic FGF induced phosphorylation of FGFR-1 and FGFR-4 to comparable extents, reflecting similar degrees of receptor activation.

The same cell lysates were then used to compare the abilities of the two receptors to induce tyrosine phosphorylation of three signaling substrates: PLCγ, ERKs, and SHC. Tyrosine phosphorylation has been shown to stimulate the enzymatic activities of PLCγ (14, 22) and ERKs (5), while phosphorylation of SHC (28) is essential to this protein's ability to complex with the *GRB2/sem-5* gene product and, presumably, activate the Ras signaling pathway (30). As shown in Fig. 3B, FGFR-1 and FGFR-4 both induced phosphorylation of PLCγ, although FGFR-1 was the more effective activator. FGFR-1 stimulation also induced phosphorylation of the 52-kDa isoform of SHC (Fig. 3C) and of

ERK2 (Fig. 3D), while FGFR-4 failed to detectably phosphorylate these substrates.

We also compared early gene responses in BaF3/FR1-C3, BaF3/FR4-19, and parental BaF3 cells following acidic FGF treatment. Expression of *c-fos* and *tis11* RNA was dramatically induced in BaF3/FR1-C3 cells by FGF, with maximal expression of both RNAs seen 30 min poststimulation (Fig. 4). By contrast, BaF3/FR4-19 and BaF3 cells failed to show any induction of these genes following FGF exposure.

Hence, the difference in mitogenic potential of FGFR-1 and FGFR-4 in BaF3 cells is paralleled by differences in the signaling potential of the activated receptors.

**Mitogenic potential of an FGFR-4/FGFR-1 chimera.** If the difference in mitogenic potential of FGFR-1 and FGFR-4 is solely due to different signaling potentials of the receptors' intracellular domains, we would expect a chimeric receptor bearing the FGFR-4 ectodomain and the FGFR-1 intracellular domain to be mitogenic. A chimeric cDNA encoding such a protein, termed FGFR-4/R1C, was constructed (see Materials and Methods), inserted into pvcos, and transfected into BaF3 cells. As shown in Table 1, this construct gave colonies upon selection with acidic FGF. One resulting colony was analyzed for growth in response to various concentrations of acidic or basic FGF. As shown in Fig. 1, acidic FGF stimulated growth of BaF3/FR4/R1C cells as efficiently as it did on BaF3 FGFR-1 cells, while basic FGF was a 10-fold weaker mitogen for BaF3/FR4/R1C cells than it was for BaF3 FGFR-1 cells. Hence, the FGFR-4 ectodomain does not impair the mitogenic potential of the FGFR-1 intracellular domain but merely changes the ligand specificity of the resulting chimeric receptor.

**FGFR-mediated protein tyrosine phosphorylation in transfected L6 myoblasts.** To determine whether differences in signaling between FGFRs are unique to the BaF3 cell system, we have further studied changes in tyrosine phosphorylation elicited by activation of FGFR-1 and FGFR-4 receptors in transfected L6 myoblasts, which express very

## A.



## B.



## C.



## D.
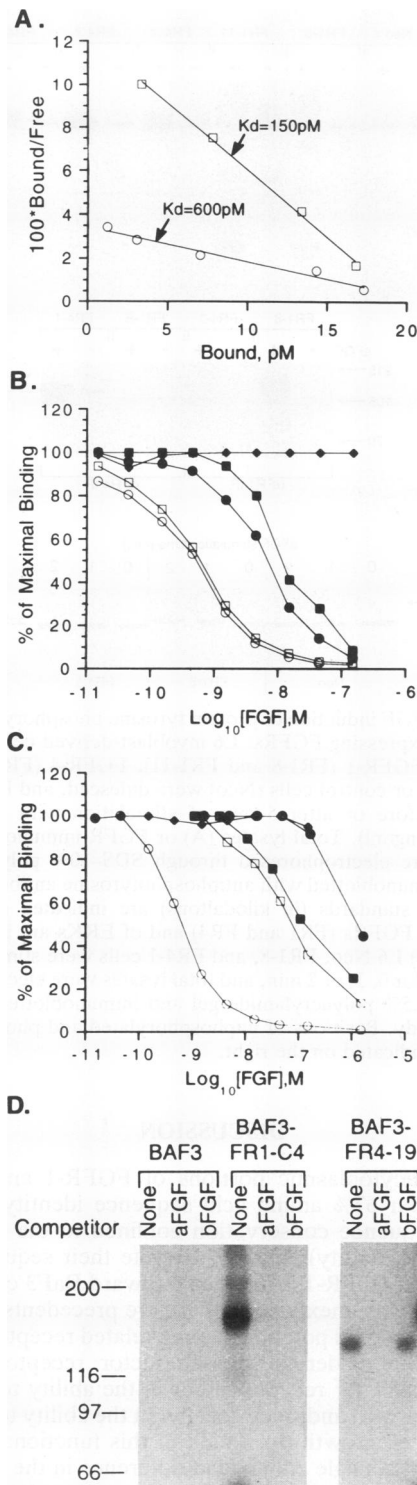


FIG. 2. Characterization of FGFRs expressed on transfected BaF3 cells. (A) Scatchard plots of acidic FGF binding to FGFRs. Different concentrations of ^{125}I-labeled acidic FGF were incubated at 4°C with either BaF3/FGFR1-A1 (□), BaF3/FGFR4.1-6 (○), or BaF3 cells, and specific binding to FGFR1 or FGFR4 was calculated as the radioactivity bound to BaF3/FGFR cells minus radioactivity bound to parental BaF3 cells. Affinity constants were derived as the negative inverse of the slope on the plot. (B and C) Competition of ^{125}I-labeled acidic FGF binding to BaF3/FGFR1-A1 (B) and BaF3/FGFR4.1-6 (C) cells by nonradioactive FGFs. ○, unlabeled acidic
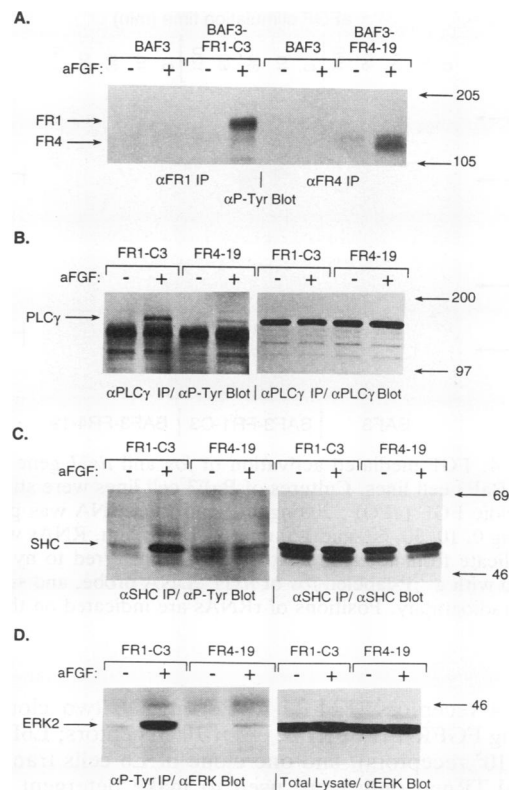


FIG. 3. Tyrosine phosphorylation of FGFRs and intracellular signaling proteins in BaF3 cell lines. Lysates were prepared from quiesced BaF3/FR1-C3, BaF3/FR4-19, and parental BaF3 cells before or after a 10-min stimulation with acidic FGF (aFGF; 200 ng/ml), and aliquots of the lysates were used for immunoprecipitation, SDS-PAGE (7.5% gel), and Western blotting. (A) FGFR tyrosine phosphorylation. Anti-FGFR-1 or anti-FGFR-4 immunoprecipitates immunoblotted with antiphosphotyrosine antibody. (B) PLCγ phosphorylation. Left, anti-PLCγ immunoprecipitates, antiphosphotyrosine immunoblot; right, reprobe of the same blot with anti-PLCγ. (C) SHC phosphorylation. Left, anti-SHC immunoprecipitates, antiphosphotyrosine immunoblot; right, reprobe of the same blot with anti-SHC. (D) ERK phosphorylation. Left, antiphosphotyrosine immunoprecipitates; right, total lysates; anti-ERK immunoblot. Positions of molecular weight markers (in kilodaltons) are indicated on the right. IP, immunoprecipitate; P-Tyr, phosphotyrosine; α, antibody; FR, FGFR.

low levels of endogenous FGFRs (24). L6 cells were also used because they give far more robust phosphorylation responses than do BaF3 cells. Receptor expression vectors were cotransfected with pLTRneo into L6 myoblasts, and G418-resistant clones were screened for FGFRs by their ability to bind ^{125}I-labeled acidic FGF (see Materials and Methods). Three clones expressing approximately $2 \times 10^5$

FGF; □, basic FGF; ■, FGF-4; ●, FGF-5; ◆, KGF. (D) Cross-linking of ^{125}I-labeled acidic FGF (aFGF) FGF to FGFRs. BaF3/FGFR and parental BaF3 cells were incubated with ^{125}I-labeled acidic FGF in the presence or absence of a 50-fold excess of nonradioactive acidic FGF or basic FGF (bFGF) and subsequently treated with disuccinimidyl suberate to cross-link FGFs to receptors. Cross-linked proteins were electrophoresed on an SDS–6% polyacrylamide gel and detected by autoradiography. Sizes are indicated in kilodaltons.
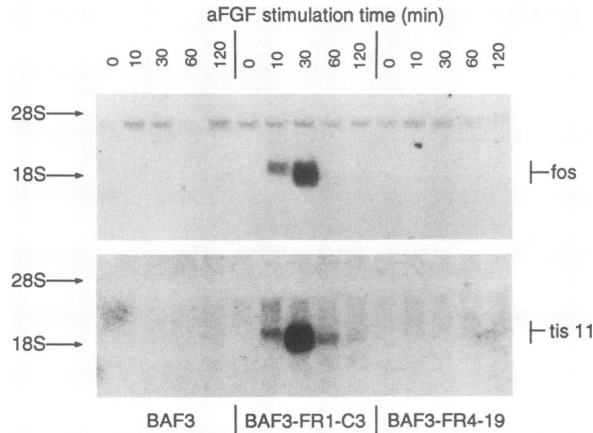
FIG. 4. FGF-mediated activation of *fos* and *tis11* gene expression in BaF3 cell lines. Cultures of BaF3 cell lines were stimulated with acidic FGF (aFGF; 200 ng/ml), and total RNA was prepared following 0, 10, 30, 60, and 120 min of stimulation. RNAs were run on duplicate formaldehyde agarose gels, transferred to nylon, hybridized with a $^{32}$P-labeled *fos* or *tis11* cDNA probe, and subjected to autoradiography. Positions of rRNAs are indicated on the left.

FGFR-4 receptors (L6FR4-1, -2, and -3), two clones expressing FGFR-1 (L6FR1-8, ~2 × 10$^5$ receptors; L6FR1-11, ~3 × 10$^5$ receptors), and one clone of L6 cells transfected with pLTRneo only were used to make detergent lysates before or after 5 min of stimulation with acidic FGF. Total lysates or immunoprecipitates with antireceptor antibodies were analyzed by Western blotting for tyrosine-phosphorylated proteins.

Cells overexpressing FGFRs showed substantial changes in protein phosphotyrosine upon FGF stimulation, whereas the control transfected cells were virtually unaffected by FGF (Fig. 5A). The most substantially phosphorylated proteins were the transfected receptors themselves, and the analysis of total lysates (Fig. 5A) and receptor immunoprecipitates (Fig. 5B) showed comparable levels of FGFR-1 and FGFR-4 autophosphorylation. FGF stimulation of cells expressing FGFR-1, but not FGFR-4, resulted in marked phosphorylation of an ~80-kDa protein (Fig. 5A). While this species is uncharacterized, it may correspond to similarly sized tyrosine-phosphorylated protein detected in FGF-stimulated fibroblasts (6, 11, 23).

FGF-induced phosphorylation of 43- and 40-kDa proteins in cells expressing FGFR-1 was noticeably more pronounced than in cells expressing FGFR-4 (Fig. 5A). These proteins have been identified as ERK-1 and ERK-2, respectively, on the basis of their molecular weights and by their recognition with anti-ERK antisera (data not shown). ERK phosphorylation is accompanied by a shift in the electrophoretic mobility of the enzyme. Activation of FGFR-1 resulted in quantitative phosphorylation of ERK-2 within 2 min (Fig. 5C) and of ERK-1 (longer exposure; not shown), whereas no detectable ERK phosphorylation was seen 2 min after FGFR-4 stimulation.

We have also assayed FGF-stimulated L6 clones for tyrosine phosphorylation of PLCγ and SHC. Stimulation of FGFR-expressing cells induced phosphorylation of both PLCγ (Fig. 6A) and SHC (Fig. 6B) proteins. However, the extent to which these substrates were phosphorylated was much greater in cells expressing FGFR-1 than those expressing FGFR-4.
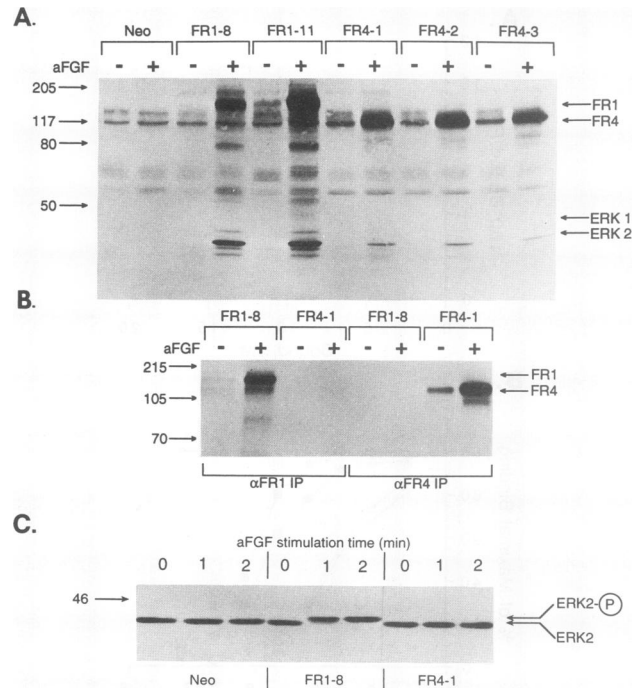


FIG. 5. FGF induction of protein tyrosine phosphorylation in L6 myoblasts expressing FGFRs. L6 myoblast-derived cell lines overexpressing FGFR-1 (FR1-8 and FR1-11), FGFR-4 (FR4-1, FR4-2, and FR4-3), or control cells (Neo) were quiesced, and lysates were prepared before or after 5 min of stimulation with acidic FGF (aFGF; 100 ng/ml). Total lysates (A) or FGFR immunoprecipitates (IP) (B) were electrophoresed through SDS–10% polyacrylamide gels and immunoblotted with antiphosphotyrosine antibody. Molecular weight standards (in kilodaltons) are indicated on the left; positions of FGFRs (FR1 and FR4) and of ERKs are indicated on the right. (C) L6 Neo, FR1-8, and FR4-1 cells were stimulated with acidic FGF for 0, 1, or 2 min, and total lysates were electrophoresed through a 7.5% polyacrylamide gel and immunoblotted with anti-ERK antibody. Positions of unphosphorylated and phosphorylated ERK2 are indicated on the right.

## DISCUSSION

The intracytoplasmic portions of FGFR-1 and FGFR-4 proteins bear 67% amino acid sequence identity, with the greatest sequence conservation confined to the kinase domains (81% identity) (27, 34). Despite their sequence similarities, only FGFR-1 is mitogenic toward BaF3 cells. While this finding was unexpected, there are precedents for differences in functional potential among related receptor tyrosine kinases. The epidermal growth factor receptor and the related gp185$^{erbB-2}$ receptor differ in the ability to stimulate fibroblast growth and, reciprocally, in the ability to stimulate lymphoid cell growth (9). Much of this functional disparity results from a single amino acid difference in the juxtamembrane regions of these receptors (8). As another example, isoforms of the TrkC neurotrophin receptor are generated by alternative RNA splicing such that the encoded proteins differ with respect to a short amino acid insertion within the kinase domain, and these isoforms differ in the ability to stimulate fibroblast growth and to induce neurite outgrowth from PC12 pheochromocytoma cells (35, 37).

FGFRs in transfected BaF3 and L6 cells have substantially different abilities to induce tyrosine phosphorylation of intracellular proteins. FGFR-1 is more potent than FGFR-4
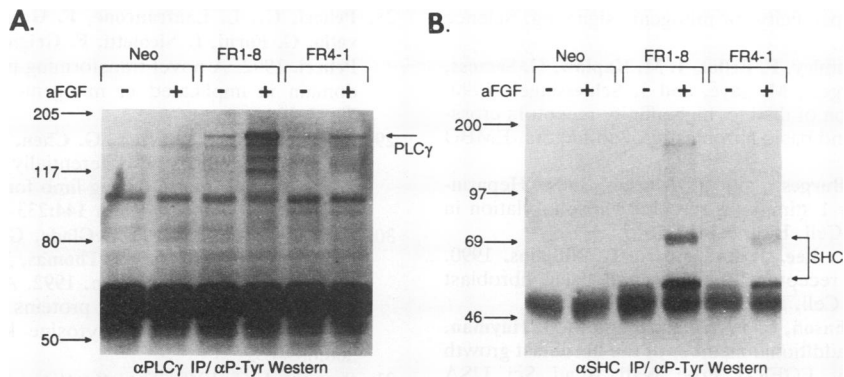
FIG. 6. FGF induction of PLCγ and SHC tyrosine phosphorylation in L6 cells expressing FGFRs. Lysates from L6-derived cell lines were prepared before or after 5 min of stimulation with acidic FGF (aFGF; 100 ng/ml) and immunoprecipitated with antibodies to either PLCγ (A) or SHC (B). Immunoprecipitates (IP) were resolved by SDS-PAGE, transferred to filters, and probed with antiphosphotyrosine (αP-Tyr) antibodies. Positions of molecular weight standards (in kilodaltons) and of PLCγ and SHC proteins are indicated. Arrows point to the 66- and 52-kDa isoforms of SHC (28), while detection of the 46-kDa isoform was obscured by the cross-reaction of the horseradish peroxidase-conjugated anti-mouse Ig with Ig heavy chains in the SHC immunoprecipitates.

at phosphorylating PLCγ, as reported previously for transfected CHO cells (36). Additionally, we have found that FGFR-4 induces much weaker phosphorylation of SHC and ERKs than does FGFR-1 and that FGFR-4 fails to induce tyrosine phosphorylation of an 80-kDa protein characteristic of the FGFR-1 response. What differences in their intracytoplasmic sequences account for the differences in FGFR-1 versus FGFR-4 signaling and function? Since tyrosine-phosphorylated epitopes on autophosphorylated receptors are sites for substrate recruitment (reviewed in reference 15), differences in the distribution of tyrosine residues along the intracytoplasmic domains of FGFRs could mediate differences in receptor function. It is noteworthy that three tyrosine residues common to the intracytoplasmic domains of FGFR-1, -2, and -3 are absent from FGFR-4 (27, 34). These differences do not directly explain why PLCγ is phosphorylated to different extents by FGFR-1 versus FGFR-4, since a site for PLCγ recruitment spans a tyrosine epitope common to the two receptors (21). FGFR-4 may lack a second of two FGFR epitopes which cooperatively bind to the two SH2 domains of PLCγ. As alternative possibilities, differences in receptor sequences could (i) influence the efficiencies with which tyrosine residues common to both receptors are autophosphorylated, (ii) affect the affinity of phosphorylated epitopes for substrates, or (iii) affect the efficiencies with which substrates recruited to common sites are subsequently phosphorylated.

Each of the FGFRs is expressed on a distinct spectrum of embryonic and adult cell types (e.g., references 27, 29, and 34), and the intracytoplasmic domain of each receptor may be optimally adapted to interface with the repertoire of intracellular signal transducers distinctive to the cells expressing that receptor. The failure of FGFR-4 to mediate growth when ectopically expressed in a hematopoietic cell line does not preclude this receptor's ability to trigger biological responses in the cell types which naturally express the receptor. We also entertain the possibility that FGFR-4 is adapted to induce minimal signaling at its natural sites of expression. Minimal signaling may allow for functional responses only in synergy with other extracellular stimuli. Alternatively, if FGFR-4 activation sequesters one or more limiting substrates without inducing their phosphorylation, this receptor could actually suppress signaling and, when

coexpressed with other FGFRs with different ligand affinity profiles, generate cells which respond to a novel repertoire of the FGFs.

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