

Receptor Binding and Mitogenic Properties of Mouse Fibroblast Growth Factor 3

MODULATION OF RESPONSE BY HEPARIN*

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Marc Mathieu, Eric Chatelain‡, David Ornitz§, Janine Bresnick, Ivor Mason¶, Paul Kiefer, and Clive Dickson||

From the Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, United Kingdom WC2A 3PX

***fgf3* has been implicated in the embryonic and fetal development of the mouse and as an oncogene in murine breast cancer. We describe a procedure to purify the product of the mouse *fgf3* gene and show it to be a potent mitogen for some epithelial cell lines. Using a receptor binding competition assay, Fgf3 was shown to bind with high affinity to the IIIb isoforms of Fgf receptor (FgFR) 1 and FgFR2 ($ID_{50} = \sim 0.8$ nM) and with a lower affinity to the IIIc variant of FgFR2 ($ID_{50} = \sim 9$ nM). No competition for the binding of ^{125}I -Fgf1 was observed for FgFR1 (IIIc), FgFR3 (IIIb and IIIc), or FgFR4. Mitogenicity assays using BaF3 cells containing individual Fgf receptors showed a pattern of response in agreement with the receptor binding results. A comparison of two mammary epithelial cell lines showed a marked difference of potency and dependence upon heparin in their response to mouse Fgf3, suggesting a complex interaction between the ligand and its low and high affinity receptors.**

The fibroblast growth factors constitute a family of nine proteins that share 35–55% amino acid identity over a core region (Refs. 1 and 2; reviewed in Refs. 3 and 4). The prototypic members, Fgf1 and Fgf2, have been ascribed a number of properties including the induction of cell proliferation, differentiation, migration, and cell survival, consistent with roles as autocrine and paracrine signaling molecules. For Fgf2 and Fgf3 there is also good evidence for the translocation of the protein directly to the cell nucleus (5–8); however, the biological significance of this event is not known. The common route for Fgf signaling is through an interaction of an extracellular Fgf with cell surface receptors (reviewed in Refs. 9 and 10). Two classes of Fgf receptor have been identified: a low affinity receptor typified by heparan sulfate proteoglycans that bind Fgfs to high capacity but seem not to signal (11) and a high affinity receptor with intrinsic tyrosine kinase activity (reviewed in Refs. 9 and 10). Four tyrosine kinase receptor genes (*fgfr1* to *fgfr4*) have been identified in mammals that encode an extra-

cellular ligand binding domain composed of two (β -form) or three (α -form) immunoglobulin-like motifs, a transmembrane segment, and a cytoplasmic portion that encompasses a tyrosine kinase domain. Fgfr1,¹ Fgfr2, and Fgfr3 but not Fgfr4 have a choice of exon encoding the second half of the third Ig loop, termed IIIb and IIIc, respectively. This alternative splicing changes the ligand binding specificity of the receptors (12–15). Both receptor classes are needed for signal transduction. The low affinity receptors are required for high affinity ligand binding and appear to facilitate the dimerization of the tyrosine kinase receptor-Fgf complexes (16–18). Dimer formation results in tyrosine autophosphorylation of the receptor providing suitable sites for second messenger interactions and consequent signal transduction (reviewed in Ref. 19).

fgf3 was identified as a proto-oncogene activated by proviral insertion in mouse mammary tumors (20, 21). Expression of *fgf3* was not detected in the normal mammary glands, suggesting that its inappropriate expression contributed to tumorigenesis. This notion gained considerable support from transgenic mouse studies (22–24). Thus, constitutive ectopic expression of *fgf3* led to abnormal mammary gland development manifest as multifocal pregnancy-sensitive epithelial hyperplasia, with the stochastic appearance of frank neoplasia. These observations prompted us to identify the receptors responsible for Fgf3 signaling and to determine which isoforms are present on mammary epithelial cells.

In this study, we describe a purification procedure for Fgf3, determine its receptor preferences, and show that these correlate with its ability to induce DNA synthesis in BaF3 cells expressing a single introduced receptor isoform. Mammary epithelial cells are shown to express receptors for and respond mitogenically to Fgf3. Using two mammary cell lines, we observed different response profiles for Fgf3 and other Fgfs that are dependent upon the concentration of heparin.

MATERIALS AND METHODS

Cell Culture—COS-1, C57MG, NIH3T3, and DMI-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. BALB/MK cells were grown in a 1:1 mixture of DMEM (without calcium) and Ham's F-12 medium containing 8% dialyzed fetal calf serum and 10 ng/ml epidermal growth factor (25). HC11 cells were grown in RMPI 1640 medium supplemented with 10% fetal calf serum, 5 μ g/ml insulin, and 10 ng/ml epidermal growth factor (26). For DNA transfections, 10 μ g of purified plasmid DNA was introduced into 5×10^6 COS-1 cells by electroporation (450 V/250 microfarads) using a Bio-Rad Gene-Pulser.

Purification of Mouse Fgf3—DMI-1 cells (27) were grown in roller bottles coated with poly-L-Lysine (Sigma). When subconfluent, the cells were changed to DMEM containing 0.1% fetal calf serum and 10 μ g/ml

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§ Supported by the Beckman Young Investigator Program and National Institutes of Health Grant CA60673. Present address: Dept. of Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, MO 63110.

¶ Present address: Division of Anatomy and Cell Biology, U.M.D.S. Guy's and St. Thomas' Hospitals, Guy's Campus, London, UK SE1 9RT.

|| To whom correspondence should be addressed. Tel.: 44-171-269-3336; Fax: 44-171-269-3094.

¹ The abbreviations used are: Fgfr, Fgf receptor; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; kbp, kilobase pair(s).

heparin (Sigma). After 48–72 h, the conditioned medium was harvested, and the following were added at the final concentrations indicated: 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 100 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM EDTA. The conditioned medium (400 ml) was gently mixed overnight at 4 °C with 80 mg of heparin-Sepharose beads (Pharmacia). The mixture was batch washed with 400 ml of isotonic phosphate-buffered saline and poured onto a Poly-Prep column (Bio-Rad). The column was washed with 20 ml of phosphate-buffered saline adjusted to 0.6 M NaCl to remove Fgf7 (see "Results"). Mouse Fgf3 was eluted in 1-ml fractions of phosphate-buffered saline containing 1 M NaCl and detected after SDS-polyacrylamide gel electrophoresis on 15% polyacrylamide gels. This procedure resulted in the isolation of a peak of mitogenic activity containing a single band of the correct size (32 kDa) as judged by silver staining. Identity to mouse Fgf3 was checked by Western blot using a polyclonal anti-peptide serum (see Fig. 1). This procedure yielded in average 10 μg of mouse Fgf3 from 4×10^8 cells.

Iodination of Fgf1, Fgf3, and Fgf7—Recombinant human Fgf1 (Bio-Tech Trade and Service), Fgf3, and recombinant human Fgf7 (Promega) were iodinated by the chloramine T method as described (28). The labeled products were separated from free iodine-125 by heparin-Sepharose chromatography and eluted with 20 mM phosphate buffer, pH 7.2, 1.5 M NaCl, 0.1% bovine serum albumin, and 1 mM dithiothreitol. Specific activities ranged from 10,000 to 30,000 cpm/ng.

RT-PCR of the Fgf Receptors—Total RNA was recovered by guanidinium thiocyanate extraction and centrifugation through cesium chloride. First strand cDNAs were synthesized using the Amersham kit with oligo(dT) as the primer. PCRs were carried out on 1 μl of template using the *Thermus aquaticus* DNA polymerase (Promega) (30 cycles with denaturation at 94 °C for 1 min, annealing at 65 °C for 2 min, and extension at 72 °C for 2.5 min). The extracellular, transmembrane, and juxtamembrane coding regions of Fgfr1 and Fgfr2 were amplified using pairs of oligonucleotides previously described (29). The PCR products were fractionated on a 1% agarose gel and stained with ethidium bromide. The expected sizes of the PCR products corresponding to the α , β , and β containing the acid box variants are 1.4, 1.1, and 1.2 kbp, respectively. To identify the presence of the IIIb and IIIc isoforms of Fgfr1, PCR using the same 5' primer as indicated above was paired with a 3' oligonucleotide specific for the antisense region of IIIb (TCG-GTCGACTCAGCGGCGTTTGAGTCCGCCAT) or IIIc (GAGTCCGATAGAGTTACCCGCCAA) domains, respectively. To distinguish the IIIb and IIIc isoforms of Fgfr2, the PCR products were digested with the restriction endonucleases *AvaI*, which cleaves specifically IIIb, and *EcoRV*, which cleaves IIIc, thereby generating a unique set of DNA fragments derived from the α , β , and β plus acid box variants (30).

Fgf Receptor Competition Binding Assay—Vectors for the expression of mouse Fgfr1 (IIIc), Fgfr2 (IIIb), Fgfr2 (IIIc), and Fgfr4 cDNAs in COS-1 cells have been described previously (29). A human Fgfr1 (IIIb) complete cDNA cloned into pBluescript KS (+) (Stratagene) was kindly provided by Dr Sabine Werner (31). The translation start was optimized according to Kozak (51) by PCR (5' oligonucleotide, tcgtctagaccATGgG-GAGCTGGAAGTGCCTCCTC and 3' oligonucleotide, tcgtctagaT-CAGCGGCGTTTGAGTCCGCCAT), and the resulting 2.2-kbp fragment was then subcloned into the *XbaI* site of the expression vector pKC3 (32). Lowercase nucleotides are added linker or mutated sequences absent in the cDNA; the start and stop codons are indicated in boldface type. Complementary cDNAs for mouse Fgfr3 isoforms were transferred as *HindIII*–*BamHI* fragments from MomFR3SV and MomFR3IIIbSV (13, 33) to the expression vector pKC3.

COS-1 cells were transfected with the appropriate Fgf receptor and seeded at 5×10^4 cells/well into 48-well tissue culture dishes pretreated with poly-L-Lysine (Sigma) as described by the manufacturer. After 48 h, the cell monolayers were washed twice with ice-cold binding medium (DMEM containing 50 mM Hepes, pH 7.4, 1 mg/ml bovine serum albumin, and 1 $\mu\text{g/ml}$ heparin) and incubated for 3 h at 4 °C with the indicated amounts of ^{125}I -Fgf1 or ^{125}I -Fgf3 in binding medium. Competition binding was performed in the presence of up to 200-fold excess of unlabeled ligand. The cell monolayers were then rinsed twice with cold binding medium, and solubilized by incubating in 0.1% SDS and 0.3 M NaOH for 30 min at 37 °C, and the associated gamma radiation was counted. To determine specific binding, the radioactivity bound to cells transfected with empty vector was subtracted from that of cells receiving Fgf receptors. The amount of ^{125}I -Fgf bound was plotted against the concentration of competitor, and the dose that inhibits the binding by 50% (ID_{50}) was then calculated.

Thymidine Incorporation Assays—Full-length cDNAs encoding the IIIb and IIIc variants of Fgfr1 and Fgfr2 were cloned into the expression vector MIRB. MIRB contains the Molony murine leukemia virus

long terminal repeat, unique *EcoRI*, *BamHI*, and *SpeI* sites followed by the IRES-NEO gene in the Bluescript KS plasmid (described in Ref. 13). The plasmid pSVFgfr1 IIIb (31) was cloned as a 2.9-kbp *BamHI*–*SpeI* fragment into the corresponding sites of MIRB. Fgfr1 (IIIc) (18) was cloned as a 3.2-kbp *EcoRI* fragment into MIRB by converting a 3' Asp⁷¹⁸ site into an *EcoRI* site and then excising with *EcoRI*. Fgfr2 (IIIb) (12) was cloned as a 2.9-kbp *BamHI* fragment into MIRB by converting a 5' Asp⁷¹⁸ site into a *BamHI* site and then excising with *BamHI*. Fgfr2 IIIc (34) was cloned into MIRB as a 3.6-kbp *SpeI* fragment by converting unique *NarI* and *XbaI* sites into *SpeI* sites.

MIRB-Fgfr plasmids were transfected into BaF3 cells and selected in the presence of 600 $\mu\text{g/ml}$ G418 (Life Technologies, Inc.). Individual clonal cell lines were isolated by limiting dilution and screened for responsiveness to Fgf1. These cell lines were used in quantitative proliferation assays, measuring [³H]thymidine incorporation into DNA as described previously (13, 33, 35). Recombinant Fgf1 (provided by K. Thomas, Merck) was used as a positive control in each experiment because Fgf1 is the only Fgf ligand that can activate all splice variants of all Fgf receptors.²

BALB/MK and NIH3T3 cells were transferred to 48-well tissue culture plates (2×10^4 cells/well) in 0.5 ml of growth medium and left for 9 or 7 days, respectively, to become confluent and quiescent (29). The culture medium was then replaced with serum-free medium containing the test samples and processed as described previously (36). C57MG and HC11 cells were made quiescent by replacing the medium after 24 h of growth with DMEM containing 0.1% new born calf serum. After a further 72 h, the cells were treated with the test samples in fresh medium containing 0.1% serum for 22 h. [³H]Thymidine incorporation assays were performed as described previously (36).

RESULTS

Purification of Mouse Fgf3—As a source of mouse Fgf3, we have used the conditioned culture medium from DMI-1 cells (27). These transformed NIH3T3 cells emerged from a selection procedure in which colonies transfected with *fgf3* cDNA in a murine leukemia virus-based vector were tested for their ability to grow as anchorage-independent colonies in soft agar and in a defined medium lacking growth factors (37). The highly transformed phenotype was shown to correlate with high levels of cell-associated and secreted Fgf3 (38). Unfortunately, this cell line, like several other fibroblast lines, produces mouse Fgf7. To separate these two Fgfs we have taken advantage of their differential capacity to bind heparin-Sepharose (25). In an initial experiment, DMI-1 cell-conditioned medium was spiked with recombinant ^{125}I -Fgf7, which eluted from the heparin-Sepharose column with 0.6 M NaCl. The bound Fgf3 was subsequently eluted in phosphate-buffered saline containing 1 M NaCl, and no ^{125}I -Fgf7 was found to co-purify as judged by Western blotting of the column fractions (data not shown). However, to further assess the potential contamination of Fgf3 by Fgf7, we used a sensitive mitogenicity assay in conjunction with a neutralizing antibody to Fgf7 (monoclonal antibody 1G4 was generously provided by J. Rubin and S. Aaronson, National Institutes of Health). The results show that the Fgf7-stimulated incorporation of [³H]thymidine was inhibited to approximately 90% by the antibody, whereas no significant effect was observed on the incorporation induced by Fgf3 (Fig. 1a). When fractionated by SDS-polyacrylamide gel electrophoresis and visualized by silver staining or immunoblotting, the preparations of mouse Fgf3 yielded a major product migrating with a relative mass of 32 kDa (Fig. 1, b and c). Thus, both biochemical and bioassay procedures failed to detect Fgf7 contamination of Fgf3.

Relative Receptor Binding Affinities of Fgf3—The Fgf receptors that bind mouse Fgf3 were identified by a competition binding assay. Initially, ^{125}I -Fgf1 was used as tracer because it interacts with all known Fgf receptors. COS-1 cells expressing similar amounts of different Fgf receptors were incubated with 0.3 nM ^{125}I -Fgf1 in the absence or presence of increasing

² D. Ornitz, unpublished observation.

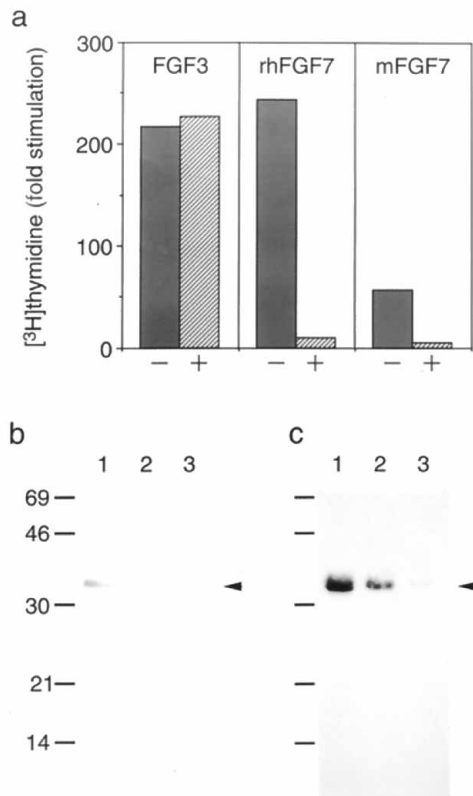


FIG. 1. Purity of mouse Fgf3. *a*, mouse Fgf3 ($FGF3 = \sim 1$ nM), recombinant human Fgf7 ($rhFGF7 = \sim 4.5$ nM), and mouse Fgf7 ($mFGF7$; partially purified from DMI-1 cells and tested at a 1/50 dilution) were added in the absence (-) or presence (+) of 5 $\mu\text{g}/\text{ml}$ of the Fgf7-neutralizing antibody 1G4 to quiescent BALB/MK cells in a mitogenicity assay. The response is plotted as the fold stimulation over antibody-treated or -untreated control cells (the antibody had no significant effect on the unstimulated cells). The mean value of duplicate determinations is shown. Silver stain (*b*) and Western blot (*c*) analyses of Fgf3 eluted from a heparin-Sepharose column loaded with DMI-1 cell-conditioned medium (see "Materials and Methods"). Fraction numbers are indicated on *top*, and the sizes of molecular weight markers (Life Technologies, Inc.) on the *left*.

amounts of Fgf3. ID_{50} values were calculated from competition binding curves as presented in Fig. 2. The results show that mouse Fgf3 effectively competes with ^{125}I -Fgf1 for binding to the IIIb and IIIc isoforms of Fgfr2 and the IIIb isoform of Fgfr1. Over the concentration range tested, which gave a molar excess of up to 133-fold, there was no competition for Fgfr1 (IIIc), Fgfr3 (IIb and IIIc), or Fgfr4. Surprisingly, the apparent affinity of mouse Fgf3 for the Fgfr2 receptor isoforms were approximately 10-fold lower than those previously found using the *Xenopus* homologue of Fgf3 (Table I and Ref. 29). This result indicated that either mouse Fgf3 has a lower intrinsic affinity for these receptors or that its binding site for the receptor diverges from that of Fgf1. To distinguish between these two possibilities, the affinity of mouse Fgf3 was determined in a homologous system using ^{125}I -Fgf3 instead of ^{125}I -Fgf1 (Fig. 3). The ID_{50} values using ^{125}I -Fgf3 as tracer were found to be 10-fold lower for Fgfr1 (IIIb) and for Fgfr2 (IIIb) but not for Fgfr2 (IIIc). Hence, mouse Fgf3 binds with a high affinity to the IIIb variants of Fgfr1 and Fgfr2 ($ID_{50} = \sim 0.8$ nM) and binds with a lower affinity to Fgfr2 (IIIc) ($ID_{50} = \sim 9$ nM) (see Table I).

Mitogenic Activity on Cells Expressing Individual Fgf Receptors—Mouse Fgf3 was compared with Fgf1 and Fgf7 over a range of ligand concentrations for their ability to stimulate DNA synthesis in BaF3 cells expressing individual Fgf recep-

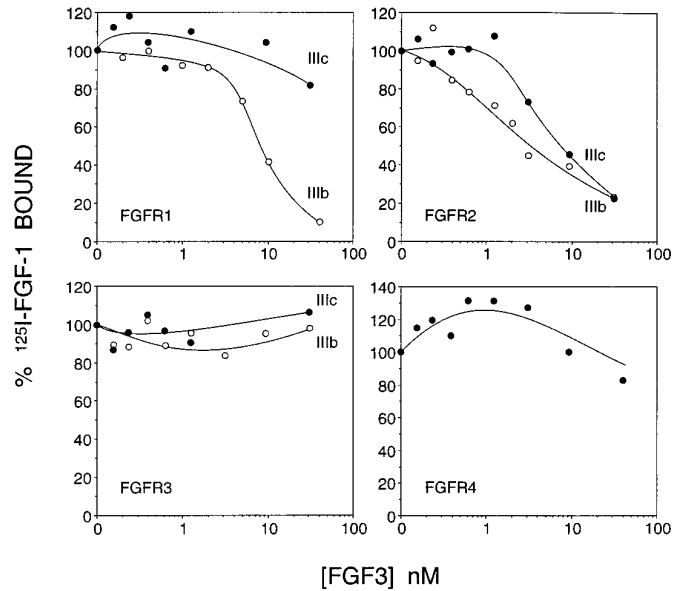


FIG. 2. Competition between Fgf3 and ^{125}I -Fgf1 for binding to different Fgf receptors. COS-1 cells expressing the IIIb (open circles) or IIIc (closed circles) isoforms of the different Fgf receptors as indicated were incubated with ^{125}I -Fgf1 in the presence of increasing concentrations of Fgf3. Cells were then washed and lysed, and specific binding was determined as described under "Materials and Methods." The calculated ID_{50} values are listed in Table I.

TABLE I
ID₅₀ values for mouse Fgf3 binding to Fgf receptors

Receptor	Tracer	
	^{125}I -Fgf1	^{125}I -mouse Fgf3
	<i>nM</i>	
Fgfr1 (IIIb)	6.0–8.1 ^a	0.7–1.3
Fgfr1 (IIIc)	>40	ND ^b
Fgfr2 (IIb)	3.4–5.8	0.6–0.9
Fgfr2 (IIIc)	7.4–11.8	9.0
Fgfr3 (IIb)	>40	ND ^b
Fgfr3 (IIIc)	>40	ND ^b
Fgfr4	>40	ND ^b

^a ID_{50} values were determined by competition binding analyses as described under "Materials and Methods" and shown in Figs. 2 and 3. The range is given when more than one determination was made.

^b ND, not determined.

tors. The results show that cells expressing Fgfr1 (IIIb) and Fgfr2 (IIb) gave the best response to Fgf3, whereas Fgfr2 (IIIc)-expressing cells were only weakly stimulated by this factor (Fig. 4). No growth stimulation occurred on BaF3 cells expressing Fgfr1 (IIIc) (Fig. 4), Fgfr3 (IIb), Fgfr3 (IIIc), and Fgfr4 (data not shown). These findings confirmed the binding specificity of Fgf3 and indicated that its interactions with Fgfr1 (IIIb) and Fgfr2 were functional. However, it is not clear at present why the mitogenic potential of Fgf3 on BaF3 cells expressing the IIIb isoforms of Fgfr1 or Fgfr2 is lower than that of Fgf1 (and Fgf7 in the latter case), because these factors have a similar affinity for these receptors (data not shown and Ref. 13). Furthermore, on a mammary epithelial cell line that naturally expresses the same receptors, Fgf3 is a more efficient mitogen than Fgf1 or Fgf7, suggesting that factors other than receptor binding affinity influence the activity of Fgfs (see below).

Modulation of Mitogenic Activity by Heparin on Mammary Epithelial Cells—During the development of the purification procedure, we found that mouse Fgf3 is a potent mitogen for the keratinocyte cell line BALB/MK (Fig. 1a). However, because of its involvement in breast cancer, we were particularly interested to assess the activity of Fgf3 on mammary epithelial

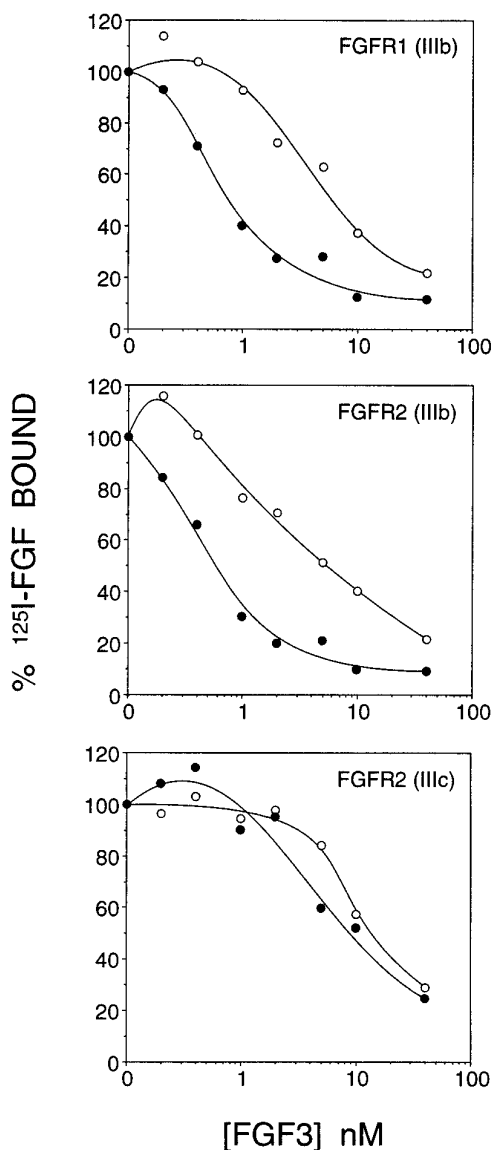


FIG. 3. Competition between Fgf3 and ¹²⁵I-Fgf1 or ¹²⁵I-Fgf3 for receptor binding. COS-1 cells expressing the indicated isoforms of Fgfr1 and Fgfr2 were incubated with ¹²⁵I-Fgf1 (open circles) or ¹²⁵I-Fgf3 (closed circles) in the presence of increasing concentrations of Fgf3. Cells were processed as described in Fig. 2. The calculated ID₅₀ values are listed in Table I.

cells. Fgf3 was indeed found to be mitogenic for the two mouse mammary cell lines, C57MG and HC11 (Fig. 5 top panels). On HC11 cells, Fgf3 was a potent inducer of DNA synthesis with an ED₅₀ (concentration eliciting 50% of the maximum response) of ~0.1 nM, whereas on the C57MG cell line the ED₅₀ was above 1 nM. Interestingly, heparin, a glycosaminoglycan that can mimic the Fgf low affinity receptors (see introduction) enhanced by more than 10-fold the potency of Fgf3 on HC11 cells (Fig. 5 right panels) while diminishing significantly the response of C57MG cells to this factor at concentrations above 2 μg/ml (Fig. 5 left panels). We also found that heparin differentially modulated the mitogenic effect of various Fgfs on the same cell line (Fig. 5 lower panels). On HC11, for instance, heparin enhanced the response to Fgf3 at all the doses tested but with a maximum effect at around 2 μg/ml. Higher concentrations of heparin were needed to increase the mitogenic activity of Fgf1, with a 3-fold enhancement at 100 μg/ml. In contrast, the response to Fgf7 was progressively inhibited at concentrations of heparin exceeding 0.5 μg/ml.

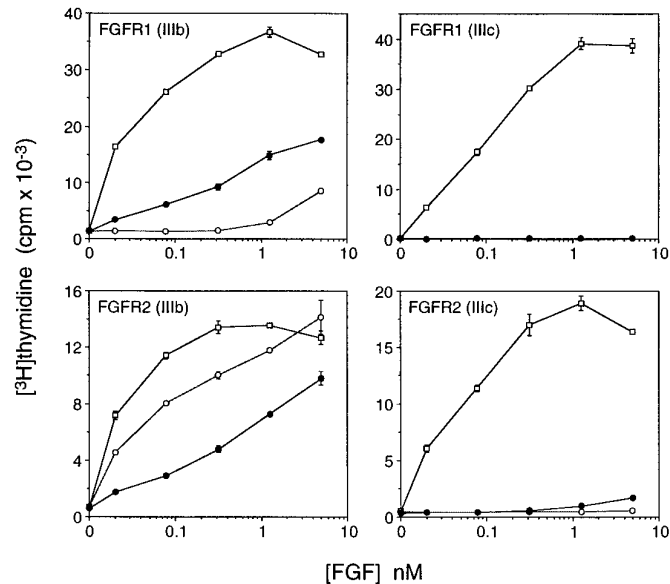


FIG. 4. Fgf3 induction of DNA synthesis in Fgf receptor expressing BaF3 cells. BaF3 cells expressing the indicated isoforms of Fgfr1 and Fgfr2 were used to compare the mitogenic activity of Fgf1 (open squares), Fgf3 (filled circles), and Fgf7 (open circles) in the presence of 2 μg/ml heparin. The mitogenic response, determined by [³H]thymidine incorporation, is expressed as the mean value ± S.D. of duplicate assays. These data are representative of two independent experiments.

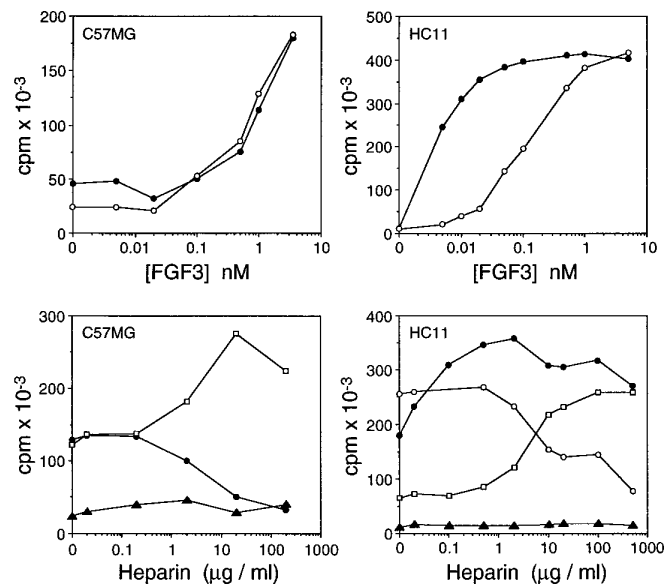


FIG. 5. Effect of heparin on the induction of DNA synthesis by Fgfs. In the top panels (C57MG and HC11 as indicated), quiescent cell cultures were treated with different concentrations of Fgf3 in the absence (open circles) or presence (filled circles) of 2 μg/ml heparin and [³H]thymidine incorporation measured as described. In the lower left panel, quiescent C57MG cells were treated or not (filled triangles) with 0.1 nM Fgf1 (open squares) or 1 nM Fgf3 (filled circles) in the absence or presence of increasing heparin concentrations as indicated. In the lower right panel, quiescent HC11 cells were treated or not (filled triangles) with 0.1 nM Fgf1 (open squares), Fgf3 (filled circles), or Fgf7 (open circles) in the absence or presence of increasing heparin concentrations as indicated. The mean value of duplicate determinations is shown.

The differential sensitivity of the two mammary cell lines toward Fgf3 could reflect a difference in the expression of its high affinity receptors. To gain some insight into the Fgf receptor repertoire of these and other Fgf3-responsive cell lines, total cell RNA was prepared and used for RT-PCR analyses with receptor-specific oligonucleotide primers. The entire ex-

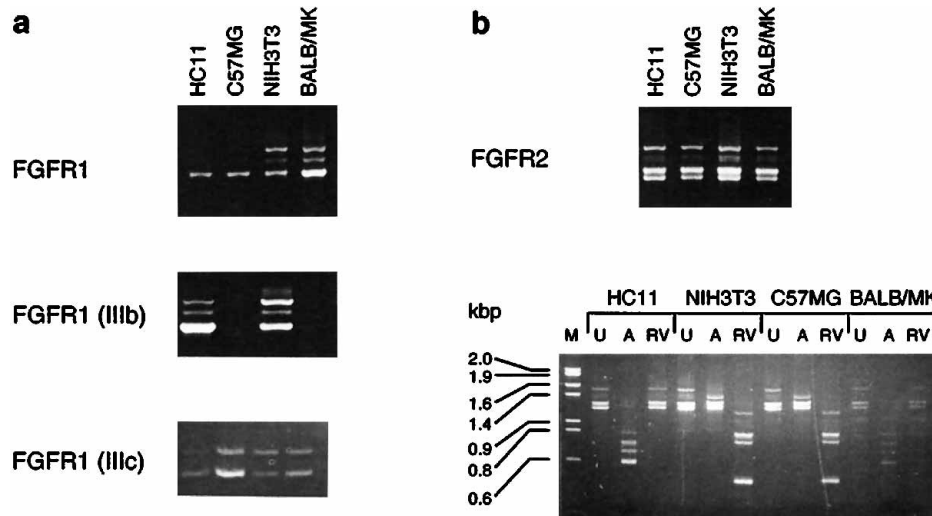


FIG. 6. **Fgf receptor expression by Fgf3-responsive cell lines.** cDNAs from HC11, C57MG, BALB/MK, and NIH3T3 cells were used for PCR amplification of the extracellular domain using primers specific for FgfR1, FgfR1 (IIIb), and FgfR1 (IIIc) (a) or FgfR2 (b) as indicated. The ethidium bromide-stained agarose gels show three prominent PCR products consistent with the sizes for the α , β , and β plus acid box variants. FgfR2 PCR products were digested or not (L) with enzymes that uniquely digest the IIIb or IIIc exon; *AclI* (A) cuts in IIIb and *RvaI* (RV) in IIIc, respectively (30). The labels NIH3T3 and BALB/MK in panel a and top of panel b have been inadvertently switched in this figure.

tracellular domain of each Fgf receptor was amplified, and different splice variants (α and β forms) were detected by ethidium bromide staining of the PCR products fractionated on agarose gels (Fig. 6, a and b). IIIb and IIIc variants of FgfR2 were distinguished by digesting the PCR products with restriction endonucleases, which cleave exclusively one or the other variant (Fig. 6b and Ref. 30). For the corresponding isoforms of FgfR1, a different approach was needed that relied on one primer hybridizing specifically to the IIIb or IIIc spliced isoform (Fig. 6a). Three size variants of FgfR1 and FgfR2 were detected in all the cell lines tested. Previous sequence analysis of FgfR2 PCR products has shown these forms represent the α , β , and β with acid box receptor variants (29). The results show that HC11 and BALB/MK express the IIIb and IIIc isoforms of FgfR1 and FgfR2 (IIIb), whereas C57MG and NIH3T3 express only the IIIc variant of these receptors. Thus, the greater sensitivity of HC11 toward Fgf3 in the proliferation assay can be explained by the presence of two receptor isoforms with an approximately 10-fold greater affinity for Fgf3 (see Table I).

The mitogenic potential of Fgf3 was then compared with that of Fgf1 and Fgf7 on the mammary cell lines (Fig. 7). Fgf1 gave a similar response on both cell lines ($ED_{50} = \sim 0.1$ nM), whereas Fgf7 induced DNA synthesis only in HC11 cells ($ED_{50} = \sim 0.2$ nM), as expected from the previous receptor expression study. Fgf1 showed a higher efficiency than Fgf3 in triggering a mitogenic response in C57MG cells ($ED_{50} = \sim 0.1$ nM versus 1 nM), probably as a result of having a higher affinity than Fgf3 for FgfR2 (IIIc) ($ID_{50} = \sim 2$ nM versus 9 nM; Table I and data not shown), of having a broader range of Fgf receptor interactions, and of being potentiated by heparin, which was included in this assay. On the contrary, the dose-response curves obtained with HC11 cells indicated that Fgf3 was more efficient than the other Fgfs in stimulating DNA synthesis ($ED_{50} = \sim 0.01$ nM). As HC11 cells express FgfR2 (IIIb), which has a similar affinity for the three Fgfs tested (Table I and Ref. 13), the variation in potency can be explained in this case by the use of heparin at a concentration of 2 μ g/ml, which was found to be optimal for Fgf3 but not for Fgf1 and Fgf7 (see Fig. 5). However, the maximum responses obtained with these Fgfs were in the same range.

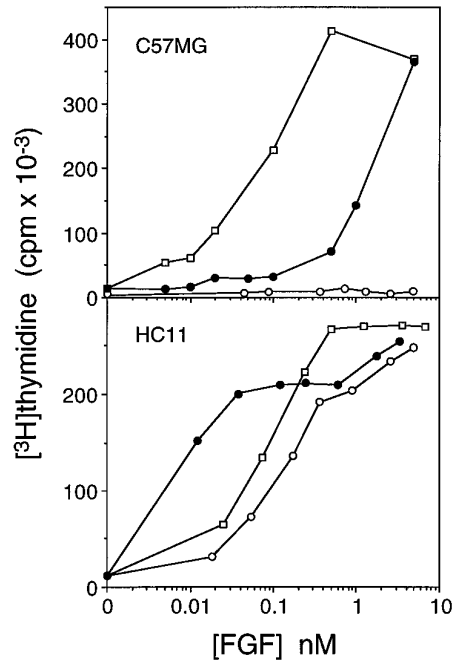


FIG. 7. **Comparative mitogenic activity of Fgf1, Fgf3, and Fgf7.** Quiescent C57MG or HC11 cells were treated with increasing concentrations of Fgf3 (filled circles), Fgf1 (open squares), or Fgf7 (open circles) in the presence of 2 μ g/ml heparin, and the stimulation of DNA synthesis was measured by the incorporation of [3 H]thymidine as described under "Materials and Methods." The mean value of duplicate determinations is shown. Results are representative of at least two experiments.

DISCUSSION

The results described herein show that biologically active mouse Fgf3 can be purified from culture supernatants of NIH3T3 cells engineered to secrete high levels of this factor. Immunological and bioactivity criteria were used to show that differential salt elution from a heparin-Sepharose column was effective in separating a mixture of Fgf7 and Fgf3 that occurs in the conditioned medium of these cells. In previous studies, recombinant Fgf3 made in bacterial or baculovirus expression

systems was found to be mostly insoluble. However, the small amount of soluble recombinant *Fgf3* showed no mitogenic activity on cells that we show here to be responsive to *Fgf3*.³ Using a cell-free translation system we were able to make biologically active *Fgf1*, *Fgf4*, and *Fgf7*, but *Fgf3* gave equivocal results (39–41). This could have resulted from the use of inappropriate assay cells, or alternatively the *Fgf3* was inactive; the studies reported here suggest the latter explanation.

The competition for receptor binding experiments (Figs. 2 and 3) clearly show that mouse *Fgf3* has the highest affinity for the IIIb isoforms of *FgfR1* and *FgfR2* and a 10-fold lower affinity for *FgfR2* (IIIc). There was no detectable competition for ¹²⁵I-*Fgf1* binding to *FgfR1* (IIIc), *FgfR3* isoforms, or *FgfR4*. It is also worth noting that we detected no significant differences between the binding of *Fgf3* to the α and β isoforms of *FgfR2* (data not shown). The lower apparent affinity of mouse *Fgf3* for *FgfR2* (IIIc) was observed using either ¹²⁵I-*Fgf1* or ¹²⁵I-*Fgf3* as tracer, indicating that mouse *Fgf3* has indeed a lower intrinsic affinity for this receptor isoform. The relative binding affinities correlated well with the mitogenic activity of *Fgf3* on BaF3 cells expressing single isoforms of the various *Fgf* receptors (Fig. 4) and are consistent with the mitogenic potential of *Fgf3* for different cell lines that express combinations of these *Fgf* receptors (Figs. 5 and 7). In contrast to mouse *Fgf3*, the *Xenopus* homologue was previously shown to have a similar high affinity for the IIIb and IIIc isoforms of *FgfR2* and, as expected, is a more potent mitogen for cells expressing *FgfR2* (IIIc) (Refs. 29 and 36 and data not shown).

Analysis of the mitogenic potential of *Fgf3* on two mammary epithelial cell lines showed that C57MG cultures required at least 10 times more ligand compared with those of HC11 to give a half-maximum response (Fig. 5). This difference in sensitivity can be largely explained by the expression on HC11 cells of the IIb variants of *FgfR1* and *FgfR2*, which show a higher affinity toward *Fgf3* than that of the IIIc variants found in C57MG cells (Fig. 6). An unexpected observation was the opposite effect of heparin on the mitogenic response elicited by *Fgf3* on the two cell lines: heparin progressively increased the potency of *Fgf3* on HC11 cells but decreased it on C57MG cells (Fig. 5). This differential modulation by heparin does not simply correlate with the presence or absence of the IIIb or IIIc variants of the *FgfR1* and *FgfR2* receptors. For instance, NIH3T3 cells are similar to C57MG in that they express the *Fgf* receptor IIIc isoforms; however, they show an enhanced response to *Fgf3* in the presence of heparin (data not shown). Furthermore, we found that heparin also differentially modulated the effect of other *Fgfs* on the same cell line. For example, heparin decreased *Fgf7* mitogenic activity on HC11 cells, whereas it potentiated *Fgf1* and *Fgf3*, albeit at different concentrations. Recently, a similar observation was reported by others using BALB/MK cells (42). Thus, different *Fgfs* may require distinct combinations and/or concentrations of low and high affinity receptors to achieve an optimal signaling effect. Response potentiation by heparin has been proposed to occur through the stabilization of the *Fgf* molecule and of *Fgf*-*Fgf* receptor complexes (18, 43–46). Oligomerization of *Fgfs* by heparin may further increase the affinity of *Fgf* for the *Fgf* receptor and results in dimerization and activation of the *Fgf* receptor (16, 17, 35). However, the mechanism of inhibition by heparin as shown here and by others (42) suggests a more complex role for the low affinity receptors in modulating *Fgf* responses.

The receptor specificity of *Fgf3* encompasses that found for *Fgf7* (13–15). It is therefore interesting that in some cases

where *Fgf7* has been implicated as a mesenchyme-secreted growth/differentiation factor acting on the adjacent epithelium, such as the prostate and seminal vesicle, *fgf3* ectopically expressed as a transgene has been shown to act as an oncogene (22, 24, 47–49). A similar situation may also exist for the mammary gland where the high affinity receptors for *Fgf3* are present and provide an explanation for its role in virally induced breast cancer. Thus, virally mediated activation of *Fgf3* directly in the epithelial cells could result in autocrine growth stimulation (reviewed in Ref. 21). Additionally, recent evidence suggests that growth factors can protect cells from undergoing apoptotic death, a process that occurs throughout the mammary gland after lactation. Therefore, *Fgf3* may act in a dual capacity to facilitate the retention and accumulation of cells susceptible to further somatic mutation. The presence of *Fgfs* and *Fgf* receptors in the mammary gland (50)⁴ also implies that the normal development and differentiation of the mammary gland during pregnancy and lactation may involve steps mediated by members of the *Fgf* family. We are presently investigating this possibility to gain further insight into the role of *Fgfs* in normal breast development.

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REFERENCES

- Miyamoto, M., Naruo, K., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. (1993) *Mol. Cell. Biol.* **13**, 4251–4259
- Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H., and Matsumoto, K. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8928–8932
- Goldfarb, M. (1990) *Cell Growth & Differ.* **1**, 439–445
- Burgess, W. H., and Maciag, T. (1989) *Annu. Rev. Biochem.* **58**, 575–606
- Baldin, V., Roman, A.-M., Bosc-Bierne, I., Amalric, F., and Bouche, G. (1990) *EMBO J.* **9**, 1511–1517
- Bouche, G., Gas, N., Prats, H., Baldin, V., Tauber, J.-P., Teissie, J., and Amalric, F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6770–6774
- Acland, P., Dixon, M., Peters, G., and Dickson, C. (1990) *Nature* **343**, 662–665
- Kiefer, P., Acland, P., Pappin, D., Peters, G., and Dickson, C. (1994) *EMBO J.* **13**, 4126–4136
- Jaye, M., Schlessinger, J., and Dionne, C. (1992) *Biochim. Biophys. Acta* **1135**, 185–199
- Johnson, D., Lee, P., Lu, J., and Williams, L. (1990) *Mol. Cell. Biol.* **10**, 4728–4736
- Moscattelli, D. (1987) *J. Cell. Physiol.* **131**, 123–130
- Dell, K. R., and Williams, L. T. (1992) *J. Biol. Chem.* **267**, 21225–21229
- Chellaiyah, A., McEwen, D., Werner, S., Xu, J., and Ornitz, D. (1994) *J. Biol. Chem.* **269**, 11620–11627
- Miki, T., Bottaro, D., Fleming, T., Smith, C., Burgess, W., Chan, A.-L., and Aaronson, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 246–250
- Zimmer, Y., Givol, D., and Yayon, A. (1993) *J. Biol. Chem.* **268**, 7899–7903
- Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994) *Cell* **79**, 1015–1024
- Ornitz, D., Herr, A., Nilsson, M., Westman, J., Svahn, C.-M., and Waksman, G. (1995) *Science* **268**, 432–436
- Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell* **64**, 841–848
- Ullrich, A., and Schlessinger, J. (1990) *Cell* **61**, 203–212
- Dickson, C., Deed, R., Dixon, M., and Peters, G. (1989) *Prog. Growth Factor Res.* **1**, 123–132
- Peters, G. (1991) *Semin. Virol.* **2**, 319–328
- Muller, W. J., Lee, F. S., Dickson, C., Peters, G., Pattengale, P., and Leder, P. (1990) *EMBO J.* **9**, 907–913
- Ornitz, D. M., Cardiff, R. D., Kuo, A., and Leder, P. (1992) *J. Natl. Cancer Inst.* **84**, 887–892
- Stamp, G., Fantl, V., Poulson, R., Jamieson, S., Smith, R., Peters, G., and Dickson, C. (1992) *Cell Growth & Differ.* **3**, 929–938
- Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S., and Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 802–806
- Ball, R., Friis, R., Schonenberger, C., Doppler, W., and Groner, B. (1988) *EMBO J.* **7**, 2089–2095
- Goldfarb, M., Deed, R., MacAllan, D., Walther, W., Dickson, C., and Peters, G. (1991) *Oncogene* **6**, 65–71
- Kan, M., Shi, E., and McKeenan, W. (1991) in *Peptide Growth Factors* (Barnes, D., Mather, J., and Sato, G., eds) Part C, pp. 158–171, Academic Press, New York
- Mathieu, M., Kiefer, P., Mason, I., and Dickson, C. (1995) *J. Biol. Chem.* **270**, 6779–6787
- Champion-Arnaud, P., Ronsin, C., Gilbert, E., Gesnel, M. C., Houssaint, E., and Breathnach, R. (1991) *Oncogene* **6**, 753–760

³ M. Mathieu, P. Acland, F. Fuller-Pace, M. Dixon, P. Kiefer, and C. Dickson, unpublished data.

⁴ J. Bresnick, unpublished data.

31. Werner, S., Duan, D.-S. R., de Vries, C., Peters, K. G., Johnson, D. E., and Williams, L. T. (1992) *Mol. Cell. Biol.* **12**, 82–88
32. Dixon, M., Deed, R., Acland, P., Moore, R., Whyte, A., Peters, G., and Dickson, C. (1989) *Mol. Cell. Biol.* **9**, 4896–4902
33. Ornitz, D. M., and Leder, P. (1992) *J. Biol. Chem.* **267**, 16305–16311
34. Mansukhani, A., Dellera, P., Moscatelli, D., Kornbluth, S., Hanafusa, H., and Basilico, C. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3305–3309
35. Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., and Leder, P. (1992) *Mol. Cell. Biol.* **12**, 240–247
36. Kiefer, P., Mathieu, M., Close, M., Peters, G., and Dickson, C. (1993) *EMBO J.* **12**, 4159–4168
37. Zhan, X., Culppepper, A., Reddy, M., Loveless, J., and Goldfarb, M. (1987) *Oncogene* **1**, 369–376
38. Kiefer, P., Peters, G., and Dickson, C. (1991) *Mol. Cell. Biol.* **11**, 5929–5936
39. Dickson, C., Fuller-Pace, F., Kiefer, P., Acland, P., MacAllan, D., and Peters, G. (1991) in *The Fibroblast Growth Factor Family* (Baird, A., and Klagsbrun, M., eds) pp. 18–26, The New York Academy of Sciences, New York
40. Dixon, M., Deed, R., Acland, P., Moore, R., Whyte, A., Peters, G., and Dickson, C. (1989) *Mol. Cell. Biol.* **9**, 4896–4920
41. Paterno, G., Gillespie, L., Dixon, M., Slack, J., and Heath, J. (1989) *Development* **106**, 79–83
42. Ron, D., Bottaro, D., Finch, P., Morris, D., Rubin, J., and Aaronson, S. (1993) *J. Biol. Chem.* **268**, 2984–2988
43. Kaplow, J., Bellot, F., Crumley, G., Dionne, C., and Jaye, M. (1990) *Biochem. Biophys. Res. Commun.* **172**, 107–112
44. Roghani, M., Mansukhani, A., Dell'Era, P., Bellosta, P., Basilico, C., Rifkin, D. B., and Moscatelli, D. (1994) *J. Biol. Chem.* **269**, 3976–3984
45. Springer, B. A., Pantoliano, M. W., Barbera, F. A., Gunyuzlu, P. L., Thompson, L. D., Herblin, W. F., Rosenfeld, S. A., and Book, G. W. (1994) *J. Biol. Chem.* **269**, 26879–26884
46. Pantoliano, M. W., Horlick, R. A., Springer, B. A., Vandyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., and Sisk, W. P. (1994) *Biochemistry* **33**, 10229–10248
47. Mason, I. (1994) *Cell* **78**, 547–552
48. Mason, I., Fuller-Pace, F., Smith, R., and Dickson, C. (1994) *Mech. Dev.* **45**, 15–30
49. Alarid, E., Rubin, J., Young, P., Chedid, M., Ron, D., Aaronson, S., and Cunha, G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1074–1078
50. Coleman-Krnack, S., and Rosen, J. M. (1994) *Mol. Endocrinol.* **8**, 218–229
51. Kozak, M. (1991) *J. Biol. Chem.* **266**, 19867–19870