A Natural Kinase-Deficient Variant of Fibroblast Growth Factor Receptor 1†

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ABSTRACT: A fibroblast growth factor receptor 1 variant missing 37 amino acids from the carboxy-terminal tyrosine kinase catalytic domain was discovered in human lung fibroblasts and several other human cell lines. The receptor variant binds specifically to acidic fibroblast growth factor but has no tyrosine kinase activity. It was found that cellular transfectants expressing the fibroblast growth factor receptor 1 variant are mitogenically inactive and ligand binding to the receptor causes neither receptor autophosphorylation nor phospholipase C-γ transphosphorylation. The fibroblast growth factor receptor 1 variant therefore represents an inactive receptor for acidic fibroblast growth factor. Since both kinase and kinase-deficient receptor forms are expressed in cells, it is conceivable that the kinase-deficient receptor plays an important role in regulating cellular responses elicited by acidic fibroblast growth factor stimulation.

Presently the family of fibroblast growth factors consists of nine different proteins. In addition to aFGF[1] and bFGF, seven other members have been identified, of which four are putative oncogene products (int-2, hst/KS3, FGF-5, and FGF-6), keratinocyte growth factor FGF-7 (Burgess & Maciag, 1989; Finch et al., 1989; Gospodarowicz et al., 1986), androgen-induced growth factor FGF-8 (Tanaka et al., 1992), and glia-activating factor FGF-9 (Miyamoto et al., 1993). aFGF is mitogenic for fibroblasts as well as endothelial cells and several other types of mesoderm- and neuroectoderm-derived cells (Burgess & Maciag, 1989). In addition to its mitogenic and chemotactic activities, aFGF is also known to promote cellular differentiation *in vitro*.

FGFs mediate their functions through high-affinity binding to and activation of cellular receptors. FGF receptors are tyrosine kinases (Lee et al., 1989) and themselves represent a family of proteins encoded by at least four separate genes. Diversity in FGF signaling is determined in part by the affinity of specific ligand−receptor pairs (Mason, 1994). Alternative splicing in the FGFR ligand binding domain generates additional receptor isoforms with novel ligand affinities. The consequence is the creation of complex combinatorial signaling pathways through which the ligands can regulate cellular growth and differentiation. A lower affinity class of binding sites for FGFs has also been identified which are heparan sulfate proteoglycans found on the cell surface and in the extracellular matrix (Rapraeger et al., 1991; Yayon et al., 1991; Ruoslahti & Yamaguchi, 1991). Apparently the low-affinity proteoglycan receptor is an essential accessory molecule required for binding of aFGF to the high-affinity tyrosine kinase FGFR and subsequent receptor dimerization, which is required for the activation of the receptor's tyrosine kinase activity (Burgess & Maciag, 1989; Pantoliano et al., 1994; Spivak-Kroizman et al., 1994). Although a great deal of information has been accumulated for other growth factor receptors of the tyrosine kinase type, at present the signaling pathways are poorly understood for FGFR. Treatment of cells with FGF leads to increased intracellular pH and Ca^{2+} levels, hydrolysis of phosphoinositides, phosphorylation of cellular proteins, and transcription of a subset of cellular genes, including c-*myc* and c-*fos*. Depending on the cell type, exposure to FGFs ultimately leads to proliferation, differentiation, inhibition of differentiation, or maintenance of a differentiated phenotype (Mason, 1994). The different FGFR genes have markedly different patterns of expression during development, indicating that receptor diversity plays a critical role in the action of FGF. An apparent involvement of FGFR3 in bone growth and development has been indicated recently, and mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia (Shiang et al., 1994).

The intracellular domains of the FGFRs dictate their functional capacity. Binding of heparin-complexed FGF to the high-affinity FGFR leads to dimerization of two receptor molecules (Pantoliano et al., 1994; Spivak-Kroizman et al., 1994), auto- and transphosphorylation of cytoplasmic tyrosine residues on the receptor, recruitment and tyrosine phosphorylation of several cytoplasmic polypeptides, and upregulation of immediate early genes (Klint et al., 1995; Mason 1994; Mohammadi et al., 1996; Zhan et al., 1993a,b). Alternative splicing also results in protein isoforms that have a different structure in the intracellular kinase domain (Shi et al., 1993), analogous to the FGFR isoforms that differ in the extracellular part of the molecule.

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[1] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; DME, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FGFR1′, fibroblast growth factor receptor 1′; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Ig, immunoglobulin-like domain; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLC-γ, phospholipase C-γ; PMSF, phenylmethanesulfonyl fluoride; RT, reverse transcription; SH2, Src homology 2; TBS, Tris-buffered saline.

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The signaling and biological responses elicited by different FGFRs differ substantially. For example, aFGF stimulation of FGFR1 and FGFR4 results in a different pattern of tyrosine-phosphorylated proteins. Despite these different patterns, however, activation of both receptor types leads to increased cellular DNA synthesis (Vainikka et al., 1994).

In the course of our studies, which aimed at the analysis of the expression of FGFR1 in several human cell lines using the RT-PCR methodology, we discovered that, next to the expected cDNA of FGFR1, an additional smaller cDNA is amplified that is missing 111 base pairs from the FGFR1 cDNA. The alternate FGFR1′ mRNA encodes a transmembrane receptor for aFGF that is missing 37 amino acids in the carboxy-terminal tyrosine kinase catalytic domain. We describe here the characterization of the FGFR1′ variant, which represents a natural dominant negative receptor for aFGF.

EXPERIMENTAL PROCEDURES

Isolation of RNA, RT, and PCR. Total cytoplasmic RNA was isolated from cultured human lung fibroblasts (established from a lung biopsy of an individual with no detectable lung disease), human monocyte-like U937 cells (UCSF Cell Culture Facility, San Francisco, CA), normal human breast epithelial cells 184 (obtained from Dr. Martha Stampfer, Lawrence Berkeley Laboratories), and two human breast cancer cell lines, ZR-75-1 and T-47-D (ATCC, Rockville, MD). Reverse transcription was carried out with an oligo(dT) primer (Pharmacia LKB, Piscataway, NJ). PCR amplification was performed with the 5′ sense primer 5′TACAAGATGAAGAGTGGTAC3′ (bp 1276−1295) and the 3′ antisense primer 5′TCAGCGGCGTTTGAGTCCGCCATT3′ (bp 2556-2533). For the expression of FGFR1 intracellular domains in baculovirus, PCR amplification was carried out with the 5′ sense primer 5′GCATGAGGATCCATGGACTACAAGGACGACGATGACAAGCTTGCAGGGGTCTCTGAGTATGAGCTT3′ and the 3′ antisense primer 5′TTGATGTCTAGATCAGCGGCGTTTGAGTCCGCCATT3′. A *Bam*HI site, a FLAG peptide sequence, and an enterokinase cleavage site were included in the sense primer. An *Xba*I site was included in the antisense primer. The two primers recognize the flanking sequence of a 1122-bp cDNA encoding the intracellular domain of FGFR1. The PCR reaction mix consisted of 35 μL of sterile double-distilled water, 5 μL of 10× reaction buffer, 1 μL of 10 mM dNTPs mixture, 0.25 μL of Taq polymerase (5 units/μL) (Promega, Madison, WI), 0.5 μL of each of the primers (20 μM), and 5 μL of single-stranded cDNAs. In the case where radioactive PCR was performed, 10^6 cpm of ^{32}P-end-labeled 5′ primer was added to the reaction. Each of the 35 cycles performed in a PCR thermocycler (Perkin Elmer, Norwalk, CT) included denaturation at 94 °C for 1 min, reannealing of primers and fragments at 55 °C for 1 min, and primer extension at 72 °C for 3 min, and final extension of primer at 72 °C for 10 min. The resultant cDNA products were analyzed on a 3% agarose gel and stained with ethidium bromide, or in the case of radioactive PCR exposed to X-ray film and the radioactive cDNA bands measured in a scintillation counter. For the expression of full-length FGFR1, the PCR amplification of the single-stranded cDNA mixture was carried out with the 5′ sense primer 5′TCATGCAAGCTTATGTGGAGCTGGAAGTGCCT3′ and the 3′ antisense primer 5′TTGATGTCTAGATCAGCGGCGTTTGAGTCCGCCATT3′, which include *Hin*dIII and *Xba*I adapter sites at their ends, respectively.

Ribonuclease Protection Assay. An RNA probe complementary to part of the coding region of the FGFR1 gene (bp 2122-2556) was generated by transcription from the pGEM-3Z plasmid (Ambion, Austin, TX) in the presence of [α-^{32}P]-UTP (Amersham, Arlington Heights, IL). RNA probe synthesis was carried out with T7 polymerase and the RNA probe was purified on a 5% polyacrylamide gel. The labeled RNA probe (10^5 cpm) was then coprecipitated with 30 μg of total RNA from human lung fibroblasts or control yeast tRNA (Ambion) and the RNA pellet was dissolved in 20 μL of hybridization buffer (Ambion). After the sample was heated at 90 °C for 10 min, hybridization was performed at 42 °C for 12 h. Subsequently, 298 μL of RNase digestion buffer was added along with 2 μL of RNase A/T1 mixture (Ambion). After a 30-min incubation at 37 °C, 6.6 μL of 10% SDS and 4.4 μL of proteinase K (10 mg/mL) were added and the incubation was continued for 15 min at 42 °C. The mixture was then extracted with phenol/chloroform, and the protected RNA was precipitated by adding 2.5 volumes of ethanol. The pellet was dissolved in 10 μL of loading buffer (Ambion), denatured at 95 °C for 5 min, and resolved on a 5% polyacrylamide gel. The dried gel was exposed to X-ray film.

Expression of Recombinant FGFR Intracellular Domains in Insect Cells. FGFR1 intracellular domain cDNAs amplified by RT-PCR (see above) were cloned into the pCRII vector (Invitrogen, San Diego, CA), and then subcloned into baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA) using *Bam*HT and *XBa*I sites. The identities of the inserts were checked by double-stranded DNA sequencing using the dideoxy chain-termination method. Recombinant viruses encoding FGFR1 intracellular domain proteins were prepared by using the BaculoGold kit (Pharmingen) according to the manufacturer's directions. Sf9 cells were infected with recombinant viruses and harvested after 72 h. The cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, pH 7.5, and 10 mM $NaPP_i$, pH 7.5) on ice for 30 min. After removal of the cellular debris by centrifugation at 4 °C for 30 min (12 000 rpm), the cell lysates were diluted 20-fold in TBS (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl). The expressed FGFR1 intracellular domain proteins were purified by using an anti-FLAG M2 affinity column (IBI, New Haven, CT), concentrated, and analyzed by SDS−PAGE.

In Vitro Tyrosine Kinase Assays. Equal amounts of purified FGFR1 intracellular domain proteins (as determined by dot-blot analysis; see below) were diluted with 20 μL of buffer containing 20 mM $MnCl_2$ and 20 mM Tris-HCl, pH 7.5, and incubated at room temperature with 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) (Amersham) alone or various concentrations of ATP that was spiked with [γ-^{32}P]ATP (Amersham). The specific activity of the mix was 0.2 μCi/nmol of ATP, and the ATP concentrations used were 10 mM, 1 mM, 100 μM, 10 μM, and 1 μM. All kinase reactions were carried out for 30 min except for 1 mM ATP, where additional time points of 5 and 10 min were performed. Samples were then mixed with 25 μL of 2× SDS−PAGE sample buffer and boiled for 10 min. The samples were loaded on an SDS−12.5% polyacrylamide gel and run at 45

V overnight. The gel was fixed, vacuum-dried, and exposed to either X-ray film or a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

For the synthetic peptide substrate assay (Turck & Edenson, 1994) to 1 μg or 10 ng of lyophilized synthetic peptide substrate derived from the FGFR1 cytoplasmic domain (VIVEYASKGN) in 1 μL of 120 mM Tris-HCl, pH 8, were added 8 μL of resin-immobilized enzyme suspension in kinase buffer and 1 μL of [γ-^{32}P]ATP (0.2 μCi/nmol) to give a 10 mM ATP concentration (same as above). The mix was incubated for 30 min at room temperature. The reaction was stopped by the addition of 2 μL of acetic acid and supplemented with 5 μg of a carrier peptide (EADNDFII-PLPD), and the mix was then loaded onto a Magic Minicolumn (Promega) filled with 500 μL of a DEAE-Sephadex A25 (Pharmacia LKB) slurry in 30% acetic acid. The columns were spun at 2000 rpm, and the peptide-containing breakthrough fraction was dried, resuspended in 5 μL of 30% acetic acid, and spotted in 1-μL aliquots onto a 20 $\times$ 20 cm^2 cellulose TLC plate (J. T. Baker Inc., Phillipsburg, NJ). TLC plates were developed in water/pyridine/acetic acid/1-butanol (60:50:75:15), air-dried, and exposed to a phosphorimager (Molecular Dynamics).

Stable Transfectants. To generate stable transfectant cell lines expressing the two FGFR1 variants, RT-PCR products (see above) were cloned into the pCRII vector (Invitrogen) and then subcloned into the pRC/CMV vector (Invitrogen) using *Hin*dIII and *XBa*I sites. L6 rat myoblast cells were transfected with 2 μg of expression vector encoding either the FGFR1 or FGFR1′ variants using the lipofectin (Gibco BRL, Gaithersburg, MD) procedure. Clones were isolated after 1−2 weeks of selection in 340 μg/mL Geneticin (Gibco/BRL) and were screened for expression of FGFR1 or FGFR1′ by performing Western blot, Northern blot, and RT-PCR assays. Stable L6 transfectants were grown in DME-H-21 medium containing 340 μg/mL Geneticin, 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Binding Assay. Radioiodination of aFGF was performed as described (Kan et al., 1991). Radiolabeled aFGF (1.5 $\times$ 10^4 cpm/ng) was stored in PBS containing 1 mg/mL bovine serum albumin at −70 °C. For binding analysis 0.8 $\times$ 10^6 cells (L34 FGFR1 transfectants or S32 FGFR1′ transfectants; see above) were collected and diluted into 200 μL of binding buffer (130 mM NaCl, 50 mM HEPES, pH 7.2, 5 mM KCl, 1.2 mM $MgSO_4$, and 1 mM EDTA). ^{125}I-aFGF (4.5 $\times$ 10^4 cpm), 25 μg/mL heparin, and various amounts of cold aFGF were then added to the suspension and incubated for 30 min at room temperature. Cells were then separated from the binding reaction mix by centrifugation through a 100-μL layer of oil (*n*-butylphthalate/dinonylphthalate, 3:2) and cell-bound radioactivity was assessed in a γ-counter. Data were analyzed with the RADLIG program (Biosoft, Ferguson, MO).

Immunochemical Analyses. Proteins extracted from transfected L6 cells with lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% deoxycholate, 0.5% NP-40, 0.1% SDS, 1 mM Na_3VO_4, and 5.5 μg/mL aprotinin) were incubated with an anti-FGFR1 monoclonal antibody directed against the juxtamembrane region of the receptor (kindly provided by Dr. Kevin Peters, CVRI, UCSF). After absorption of immune complexes to protein G−agarose (Boehringer Mannheim, Indianapolis, IN) and several washes, the immune complexes were subjected to SDS−PAGE followed by transfer of the proteins to nitrocellulose membranes. The membranes were then first incubated with blocking buffer (5% nonfat milk in 20 mM Tris-HCl and 137 mM NaCl, pH 7.6) for 2 h at room temperature and subsequently incubated with anti-FGFR1 monoclonal antibody (see above) or anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) in 5% bovine serum albumin for 2 h at room temperature. Immunoglobulin bound to transferred proteins was detected with a goat anti-mouse secondary antiserum and developed using the ECL Western blotting kit (Amersham). For dot-blot analyses, serial dilutions of purified FGFR1 and FGFR1′ intracellular domain proteins were spotted onto nitrocellulose membrane and processed as described above for Western blots with the anti-FGFR1-specific monoclonal antibody.

PLC-γ Immunodetection. Transfectant L6 cells were starved overnight in serum-free DME-H-21 medium containing 0.2% bovine serum albumin and then stimulated for 15 min with 10 ng/mL aFGF (Austral, San Ramon, CA). The cells were washed with PBS, lysed (see above), and sonicated. The lysate was centrifuged for 10 min and the supernatants were incubated overnight at 4 °C with an anti-PLC-γ monoclonal antibody (Upstate Biotechnology). The immune complexes were recovered by using protein G−agarose, washed with lysis buffer, solubilized in 2$\times$ SDS sample buffer, and subjected to SDS−PAGE. Proteins were then transferred to a nitrocellulose membrane and assayed for phosphotyrosine using the above procedure.

Metabolic Labeling and Immunoprecipitation. Confluent cells were incubated overnight in DME-H-21/0.2% bovine serum albumin, washed three times with phosphate-free medium, and then incubated for 2 h in phosphate-free medium/0.1% dialyzed FCS. Cells were washed again three times with phosphate-free medium and then incubated for 2 h in phosphate-free medium/0.1% FCS in the presence of 0.5 mCi of $^{32}PO_4$ (Amersham). Thereafter cells were stimulated with 10 ng/mL aFGF (Austral) for 5 min at room temperature, the dishes were put on ice, the medium was aspirated, and the cells were washed three times with cold PBS. After the last PBS wash the dishes were placed on dry ice/methanol and 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.1, 50 mM NaCl, 30 mM $NaPP_i$, 50 mM NaF, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 1 mM PMSF, 0.1% bovine serum albumin, 0.5% NP-40, and 5 μM $ZnCl_2$) was added. The lysates were immunoprecipitated with the anti-FGFR1-specific monoclonal antibody (see above) and the immune complexes were absorbed to protein G−agarose beads. Proteins were eluted from the beads in reducing SDS sample buffer and resolved by 7.5% PAGE followed by autoradiography.

Phosphoamino acid analysis. Autophosphorylated FGFR1 was eluted out of the SDS gel after rehydration and precipitated with acetone at −20 °C for 5 h. After being washed two times with cold acetone and air-dried, the pellet was mixed with 20 μL of 6 N HCl and hydrolyzed for 1 h at 110 °C. The hydrolysate was concentrated, redissolved in 10% acetic acid that contained phosphoserine, phosphothreonine, and phosphotyrosine (Sigma, St. Louis, MO), and spotted onto a cellulose TLC plate (J.T. Baker, Inc.). High-voltage electrophoresis was carried out in pyridine/acetic acid/acetone/water (1:2:8:40) at 900 V for 1 h in the cold (Savant Instruments, Farmingdale, NY). After air-drying, the plate was developed with a 0.2% solution of ninhydrin

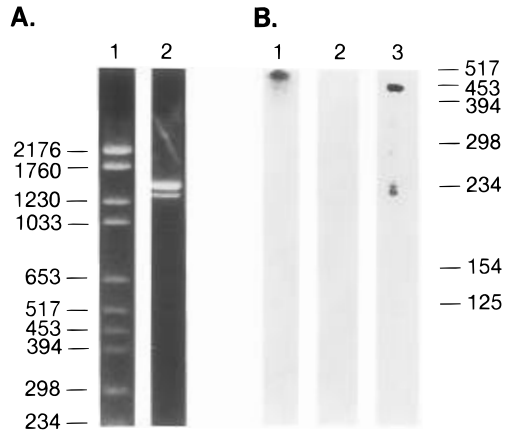


FIGURE 1: Expression of FGFR1 in human lung fibroblasts. (A) Agarose gel electrophoresis of RT-PCR amplification products. Lane 2: Two cDNA species were amplified from human lung fibroblasts with FGFR1-specific oligonucleotide primers encompassing the cytoplasmic domain of the receptor (bp 1276−2556). Lane 1: DNA markers. (B) Ribonuclease protection assay using a riboprobe complementary to bp 2122−2556 of the FGFR1 mRNA. Lane 1: Riboprobe used for the assay (514 bp). Lane 2: Yeast tRNA (control). Lane 3: Human lung fibroblast RNA. Protected are mRNA species derived from wild-type FGFR1 (435 bp) and variant FGFR1′5′ fragment (231 bp). The FGFR1′-derived 3′ fragment is not visible due to its small size (93 bp).

in ethanol to visualize the standard phosphoamino acids. Subsequently the plate was exposed to X-ray film.

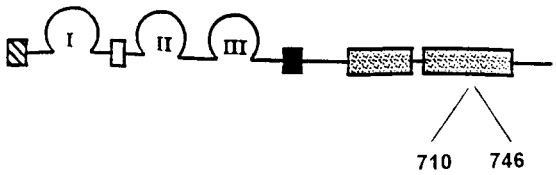
Mitogenesis Assay. On day 0, 5000 transfectant cells in 100 μL of DME supplemented with 10% FCS were seeded into each well of a 96-well plate. The following day, the cells were washed three times with DME to remove FCS, and incubated for 3 days in DME. The medium was then replaced by DME with or without 50 ng/mL aFGF (Austral) and 15 units/mL heparin and incubated for 24 h. Subsequently, 1 μCi of ^{3}H-thymidine was added to each well and incubation was continued for 6 h. After aspiration of medium, cells were washed with PBS and collected with a PHD cell harvester (Cambridge Technologies, Cambridge, MA). Filters were treated with 10% trichloroacetic acid and washed several times with water and methanol, and the incorporated radioactivity was determined by scintillation counting.

RESULTS

RT-PCR of mRNA from human lung fibroblasts (see Materials and Methods) with oligonucleotide primers specific for the intracellular domain of the human FGFR1 gene yielded two cDNAs of distinct sizes. One cDNA was of the expected size and the other novel cDNA was 111 bp smaller than the one obtained for the wild-type receptor (Figure 1A). Nucleotide sequence analysis revealed that the smaller cDNA was also derived from the FGFR1 gene (Figure 2A). Further evidence for the existence of the FGFR1 variant mRNA species was obtained by carrying out a ribonuclease protection assay with a radiolabeled RNA probe that encompassed the missing 111-bp stretch and was 514 bp long so that it could be distinguished from hybridized RNA. Figure 1B shows that next to the expected full-length FGFR1 mRNA (435 bp) the probe also protects an RNA species of 231 bp in human lung fibroblasts but not in the control sample that represents a fragment corresponding to the 5′ piece of the FGFR1 variant mRNA that is generated after RNase digestion. Due to its small amount of incorporated radioactivity, the FGFR1′ mRNA-derived 3′ fragment of 93 bp could only be detected after prolonged exposure of the gel to X-ray film (data not shown). Since the difference between the two variant mRNAs is only 111 bp, it is impossible to use Northern blot analysis for the relative quantitation of the two FGFR1 forms due to the low resolution of this method. Therefore, in order to get an estimate for the relative abundance of the two FGFR1 variant mRNAs we have used a radioactive PCR method. In this case a radiolabeled FGFR1-specific primer was included in the PCR and the resultant amplified FGFR1 cDNAs were assessed for incorporated radioactivity (data not shown). Since both mRNAs are amplified by the same primer set, this method allows one to get a good estimate of the ratio of the two FGFR1 variants. The human lung fibroblasts used in this study have a mRNA ratio of FGFR1 wild type to FGFR1′ of 5:1. Other analyses revealed that different FGFR1/FGFR1′ ratios are observed depending on the cell line. In particular we have investigated human breast cancer cell lines (ZR-75-1 and T-47-D) as well as a normal human breast epithelial cell line (AD155) and have found significant differences in the FGFR1/FGFR1′ ratios in these cells. Whereas the ratio of FGFR1/FGFR1′ mRNA was 1:5.25 in the normal breast epithelial cell line, much higher ratios were observed in the two tumor cell lines (1.85:1 and 7.33:1, respectively). Whether these differences in the FGFR1/FGFR1′ ratios correlate with cellular transformation and what effects they have on signal transduction events is currently being investigated.

As a consequence the receptor in-frame deletion mutant is missing a 37 amino acid stretch that is located in the C-terminal kinase catalytic domain of the cytoplasmic tail of the molecule (amino acids 710−746 of the 3 immunoglobulin-like domain FGFR1 form) (Figure 2B). Based on the recently published three-dimensional structure of the insulin receptor tyrosine kinase domain (Hubbard et al., 1994), the deleted stretch of amino acids is located in the C-terminal lobe of this domain. Although it is not part of the catalytic site itself, several of the deleted amino acid residues are predicted to be important in structure stabilization (Hubbard et al., 1994).

To investigate the functional consequences of the 37 amino acid C-terminal catalytic domain deletion, we expressed the cytoplasmic tails of the FGFR1 full-length form as well as the newly discovered mutant FGFR1′ form in baculovirus as FLAG fusion proteins and purified them from Sf9 cell lysates by affinity chromatography. The resultant purified cytoplasmic tails of both receptor forms were then subjected to *in vitro* kinase assays in order to determine their enzymatic activities. To assure that equal amounts of the two variant proteins were employed in the kinase assays, we carried out a dot-blot analysis with an FGFR1-specific monoclonal antibody. Kinase reactions were carried out in duplicate with resin-immobilized FLAG fusion proteins and various concentrations of ATP that was spiked with trace amounts of [γ-^{32}P]ATP, and the proteins were analyzed by SDS−PAGE followed by autoradiography (Figure 3). The cytoplasmic tail derived from the full-length FGFR1 resulted in autophosphorylation at a wide range of ATP concentrations (10 mM−1 μM), whereas the newly discovered FGFR1′ cytoplasmic form did not autophosphorylate at detectable levels



A.

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B.

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1   M W S W K C L L F W A V L V T A T L C T A R P S P T L P E Q
31  A Q P W G A P V E V E S F L V H P G D L L Q L R C R L R D D
61  V Q S I N W L R D G V Q L A E S N R T R I T G E E V E V Q D
91  S V P A D S G L Y A C V T S S P S G S D T T Y F S V N V S D
121 A L P S S E D D D D D D D S S S E E K E T D N T K P N R M P
151 V A P Y W T S P E K M E K K L H A V P A A K T V K F K C P S
181 S G T P N P T L R W L K N G K E F K P D H R I G G Y K V R Y
211 A T W S I I M D S V V P S D K G N Y T C I V E N E Y G S I N
241 H T Y Q L D V V E R S P H R P I L Q A G L P A N K T A L G
271 S N V E F M C K V Y S D P Q P H I Q W L K H I E V N G S K I
301 G P D N L P Y V Q I L K T A G V N T T D K E M E V L H L R N
331 V S F E D A G E Y T C L A G N S I G L S H H S A W L T V L E
361 A L E E R P A V M T S P L Y L E I I I Y C T G A F L I S C M
391 V G S V I V Y K M K S G T K K S D F H S Q M A V H K L A K S
421 I P L R R Q V T V S A D S S A S M N S G V L L V R P S R L S
451 S S G T P M L A G V S E Y E L P E D P R W E L P R D R L V L
481 G K P L G E G C F G Q V V L A E A I G L D K D K P N R V T K
511 V A V K M L K S D A T E K D L S D L I S E M E M M K M I G K
541 H K N I I N L L G A C T Q D G P L Y V I V E Y A S K G N L R
571 E Y L Q A R R P P G L E Y C Y N P S H N P E E Q L S S K D L
601 V S C A Y Q V A R G M E Y L A S K K C I H R D L A A R N V L
631 V T E D N V M K I A D F G L A R D I H H I D Y Y K K T T N G
661 R L P V K W M A P E A L F D R I Y T H Q S D V W S F G V L L
691 W E I F T L G G S P Y P G V P V E E L F K L L K E G H R M D
721 K P S N C T N E L Y M M M R D C W H A V P S Q R P T F K Q L
751 V E D L D R I V A L T S N Q E Y L D L S M P L D Q Y S P S F
781 P D T R S S T C S S G E D S V F S H E P L P E E P C L P R H
811 P A Q L A N G G L K R R
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FIGURE 2: Structures of FGFR1 and FGFR1′ (3 Ig form); the nucleotide sequence (bp 2215−2325) (A) and amino acid sequence (aa 710−746) (B) that are delected in FGFR1′ are indicated (italic, bold, underlined). The protein is encoded from bp 88 to 2553. I, II, and III, immunoglobulin-like domains; hatched box, signal sequence; open box, acid box; solid box, transmembrane domain; stippled box, tyrosine kinase domain.

for any of the ATP concentrations (10 mM and 100 μM), including physiological ATP amounts (Figure 3B). Similar results were obtained when both cytoplasmic tails were employed in a synthetic peptide substrate kinase assay (Turck & Edenson, 1994). In this assay equal amounts (as determined by a dot-blot assay with an anti-FGFR1-specific antibody) of the cytoplasmic domains of the receptor variants were subjected to *in vitro* kinase assays in the presence of a receptor-derived tyrosine-containing peptide (VIVEYASKGN). This assay had been developed by us earlier with the goal of mapping receptor tyrosine kinase autophosphorylation sites (Turck & Edenson, 1994). While the full-length FGFR1-derived kinase led to transphosphorylation of the FGFR1-derived peptide substrate, no phosphorylated target peptide was detected with the FGFR1′ cytoplasmic protein (Figure 3C). This kinase assay was performed by using a constant ATP concentration (10 mM) and varying the amount of substrate (1 μg and 10 ng). On the basis of the above results, we refer to the newly discovered FGFR1 variant as the kinase-deficient FGFR1′.

To analyze signal transduction events induced by aFGF binding to the two receptor variants we expressed both receptors in L6 rat myoblast cells, which do not express endogeneous FGFRs. For this purpose stable transfectants of the two receptor variants were established. Isolated clones were screened for expression of FGFR1 or FGFR1′ by performing RT-PCR (data not shown), Western blot (Figure 4), and binding assays (Figure 5). These analyses revealed that all cell lines expressed the respective receptor variants. The RT-PCR data show cDNA bands of the expected sizes (data not shown) that can be well resolved, whereas the two receptor protein bands on Western blots are indistinguishable

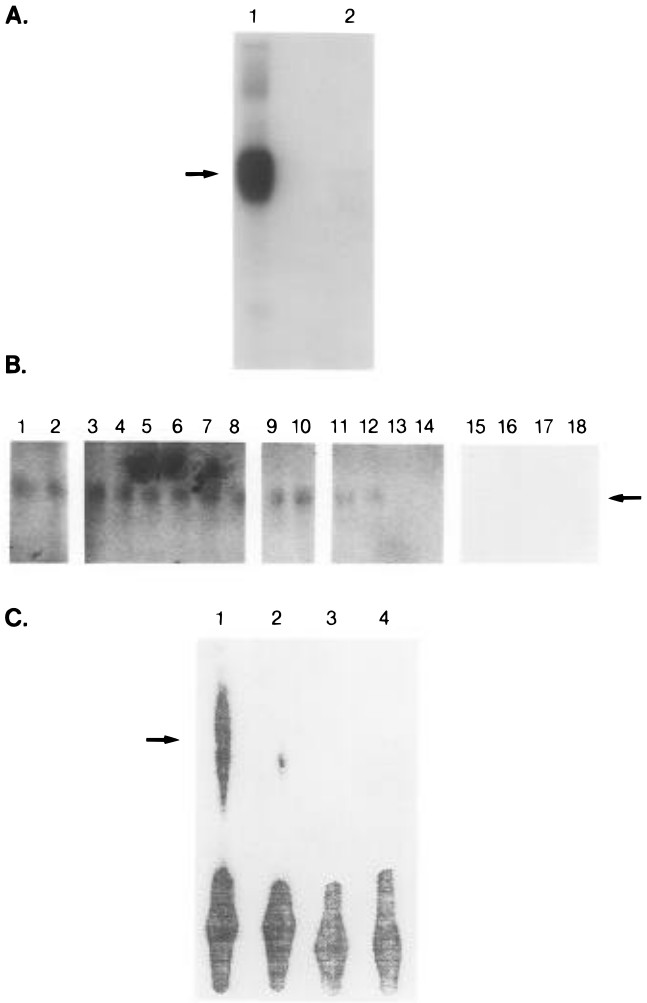

FIGURE 3: *In vitro* tyrosine kinase assays of FGFR1 and FGFR1′ cytoplasmic domains. Equal amounts of the receptor cytoplamsic domains (as assessed by dot blot analysis with an anti-FGFR1-specific monoclonal antibody) were employed as FLAG fusion proteins immobilized on anti-FLAG agarose. (A) Incubation with [γ-^{32}P]ATP alone, 30 min. Lane 1, FGFR1; lane 2, FGFR1′. (B) Reactions performed at various ATP concentrations (spiked with [γ-^{32}P]ATP) and different time points. Lanes 1−14, FGFR1: lanes 1 and 2, 10 mM ATP, 30 min; lanes 3 and 4, 1 mM ATP, 30 min; lanes 5 and 6, 1 mM ATP, 10 min; lanes 7 and 8, 1 mM ATP, 5 min; lanes 9 and 10, 100 μM ATP, 30 min; lanes 11 and 12, 10 μM ATP, 30 min; lanes 13 and 14, 1 μM ATP, 30 min. Lanes 15−18, FGFR1′: lanes 15 and 16, 10 mM ATP, 30 min; lanes 17 and 18, 100 μM ATP, 30 min. After the resin beads were washed, the fusion proteins were eluted and subjected to SDS−PAGE followed by autoradiography. The autophosphorylated wild-type receptor cytoplasmic fusion protein is marked with an arrow. (C) Synthetic peptide substrate assay using the FGFR1-derived peptide VIVEYASKGN and 10 mM ATP (see above). Lane 1, FGFR1, 1 μg of peptide; lane 2, FGFR1, 10 ng of peptide; lane 3, FGFR1′, 1 μg of peptide; lane 4, FGFR1′, 10 ng of peptide. The transphosphorylated peptide is marked with an arrow. The broad signal at the bottom of the plate is [γ-^{32}P]ATP.

due to the small difference in molecular weight of the proteins (Figure 4). Binding studies showed that the FGFR1′ kinase-deficient variant was able to bind aFGF specifically and with high affinity (Figure 5). The calculated K_d for aFGF binding to FGFR1 is 3.46 nM, whereas the one for FGFR1′ is 1.91 nM. These data are consistent with results obtained by others who also showed that kinase-deficient receptor mutants had similar ligand binding abilities as the wild-type receptors (Bellot et al., 1991; Lie et al., 1994). Even receptors without any intracellular domain have been

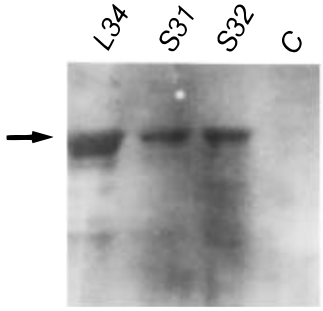

FIGURE 4: Immunoprecipitation/Western blot analysis of cellular transfectants with a FGFR1-specific monoclonal antibody. L34, FGFR1 transfectant; S31 and S32, FGFR1′ transfectants; C, transfectant with plasmid with no insert. The 125-kDa receptor bands are marked with an arrow.

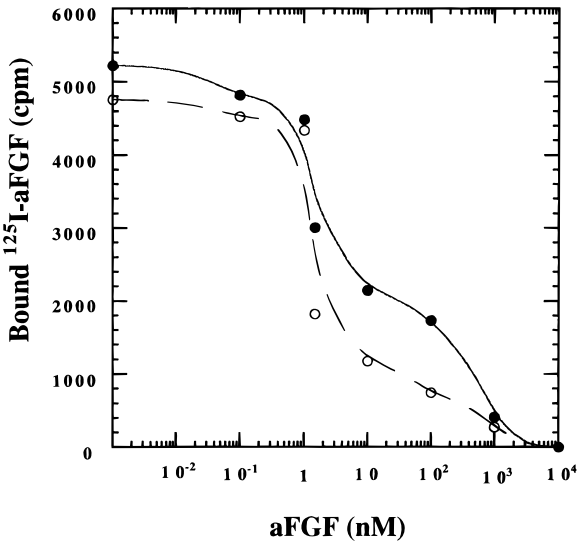

FIGURE 5: Binding assay of cellular transfectants. Specific binding of ^{125}I-aFGF to L34 (FGFR1 transfectant) (●) and S32 (FGFR1′ transfectant) (○)) cells was assessed in the presence of heparin and competition with increasing amounts of cold aFGF. Background binding at 10^4 nM of cold aFGF (1550 cpm) has been subtracted from all data points. All data points represent the average of triplicate experiments.

shown to be capable of ligand binding and subsequent receptor dimerization (Li et al., 1994).

Ligand binding to tyrosine kinase receptors leads to receptor dimerization, activation of the tyrosine kinase, and subsequent receptor auto- and transphosphorylation of tyrosine residues located in the cytoplasmic tails of the receptors (Mason, 1994; Zhan et al., 1993a; Shi et al., 1993). Whereas one study suggests that FGFR1 dimerization is brought about by a complex of one FGF and one heparin molecule (Pantoliano et al., 1994), another report presents evidence that receptor dimerization is induced by binding of two aFGF molecules that are complexed to heparin (Spivak-Kroizman et al., 1994). In order to study receptor activation we carried out *in vivo* kinase assays of the established L6 cell lines. For this purpose the cells were cultured in $^{32}PO_4$-containing medium and left unstimulated or stimulated with aFGF in the presence of heparin. Cellular lysates were prepared and subjected to immunoprecipitations with an anti-FGFR1-specific antibody. Eluates were subjected to SDS−PAGE, which revealed that whereas in FGFR1 transfectants the receptor underwent the expected tyrosine auto- and transphosphorylation after aFGF/heparin treatment of the cells, in FGFR1′ transfectant cells no phosphorylated receptor that

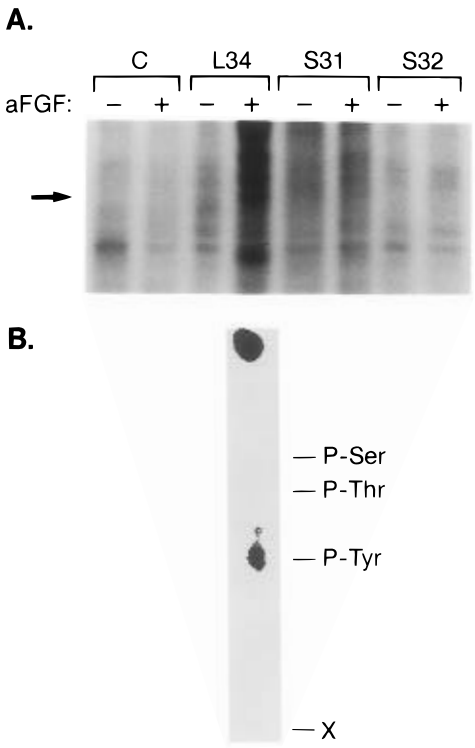

FIGURE 6: (A) *In vivo* kinase assay. Cells were metabolically labeled with $^{32}PO_4$ and were left unstimulated or stimulated with 50 ng/mL aFGF; lysates were immunoprecipitated with an anti-FGFR1 monoclonal antibody. Only aFGF-stimulated L34 cells show an autophosphorylated FGFR1 protein band, whose position is marked with an arrow. C, transfectant with plasmid with no insert; L34, FGFR1 transfectant; S31 and S32, FGFR1′ transfectants. (B) Phosphoamino acid analysis of *in vivo*-labeled FGFR1 (lane L34+) from above. The positions of the phosphoamino acid standards are indicated. The radioactive spot at the top of the plate represents free phosphate.

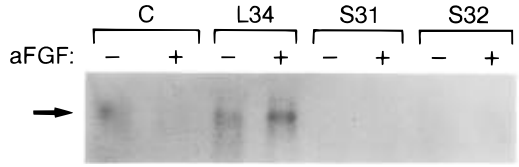

FIGURE 7: PLC-γ tyrosine phosphorylation. Cells were left unstimulated or were stimulated with 50 ng/mL aFGF; lysates were immunoprecipitated with an anti-PLC-γ monoclonal antibody. Western blots were probed with an anti-phosphotyrosine polyclonal antiserum. Only aFGF-stimulated L34 cells show a PLC-γ protein band that is tyrosine-phosphorylated. C, transfectant with plasmid with no insert; L34, FGFR1 transfectant; S31 and S32, FGFR1′ transfectants. The PLC-γ band is indicated with an arrow.

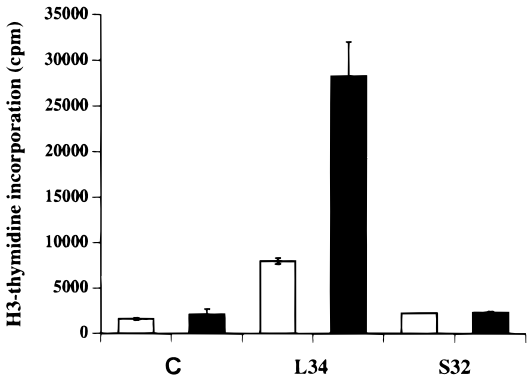

FIGURE 8: Mitogenesis assay of cellular transfectants. Cells were stimulated with 50 ng/mL aFGF (■) or left unstimulated (□) and assessed for the uptake of ^{3}H-thymidine into newly synthesized DNA. L34, FGFR1 transfectant; S32, FGFR1′ transfectant; C, transfectant with plasmid with no insert. Each analysis was carried out in triplicate.

appears as a diffuse band due to its phosphorylation was detectable (Figure 6A). Phosphoamino acid analysis of the autophosphorylated FGFR1 from Figure 6A (lane L34+) revealed only the presence of phosphotyrosine (Figure 6B). Figure 6A also shows that in addition to the phosphorylated receptor band several other phosphorylated proteins of unknown identity are detectable in immunoprecipitates of aFGF-stimulated wild-type receptor transfectants. These findings confirmed our earlier results obtained from the *in vitro* kinase assays of the cytoplasmic tails of the receptors. Apparently the 37 amino acid deletion in FGFR1′ leads to a complete inactivation of the intrinsic tyrosine kinase of the receptor.

After receptor activation and autophosphorylation, cytoplasmic proteins with phosphotyrosine-specific binding domains, such as SH2 domains, can associate with the receptor's cytoplasmic tail (Williams, 1989; Ullrich & Schlessinger, 1990; Fantl et al., 1992, 1993). PLC-γ is one such protein that has been shown to associate with FGFR1 via phosphotyrosine 766 and subsequently is tyrosine-phosphorylated itself, leading to its activation (Mohammadi et al., 1991; Peters et al., 1992). The function in the FGFR1 signaling cascade of PLC-γ association and activation is unknown. To investigate PLC-γ phosphorylation by FGFR1 and FGFR1′ we stimulated L6 transfectants with aFGF, prepared cellular lysates, and subjected them to immunoprecipitations with anti-PLC-γ-specific antibodies. The immunoprecipitated proteins were then separated by SDS−PAGE, electroblotted, and probed with anti-phosphotyrosine-specific antibodies. Figure 7 shows that only in aFGF-stimulated FGFR1 transfectants is PLC-γ phosphorylated. Studies by others have shown previously that association of PLC-γ with the activated FGFR1 is mediated by phosphorylated tyrosine 766 and that the association is necessary for PLC-γ phosphorylation and activation (Mohammadi et al., 1991; Peters et al., 1992). On the basis of these studies, we therefore conclude that PLC-γ does not associate with the FGFR1′ variant since it is not phosphorylated after aFGF stimulation of the cells.

In order to directly analyze the effects of the full-length kinase-deficient FGFR1′ variant on mitogenic function, we carried out mitogenesis assays with the transfectant cell lines. As can be seen in Figure 8, only wild-type receptor transfectants (L34) show an appreciable mitogenic response after stimulation with aFGF. Control cells (C) and variant receptor transfectants (S32), however, do not show any increase in DNA synthesis in response to growth factor stimulation.

DISCUSSION

We describe here the discovery of a novel alternate mRNA form of the human FGFR1 gene that encodes a kinase-deficient receptor for aFGF. The variant receptor was initially discovered in human lung fibroblasts but was subsequently also found in other mammalian cells. To this end we have analyzed human breast epithelial cells (184), human breast cancer cell lines (ZR-75-1 and T-47-D), and a human monocyte-like cell line (U937). In all cases both receptor variants were found by RT-PCR, albeit at different

ratios. Interestingly, in the human breast cancer cell lines the FGFR1/FGRR1′ ratios were significantly higher than in normal human breast epithelial cells. We are currently investigating these cell lines in greater detail in order to see if there is a correlation between the expression of the two FGFR1 variants and cellular signaling and transformation. The variant protein that we have discovered is missing 37 amino acids located in the C-terminal kinase catalytic domain but is otherwise identical with the wild-type receptor. We have shown that the small deletion in the kinase catalytic domain does not affect aFGF binding in cellular transfectants.

Signal transduction mechanisms of tyrosine kinase receptors are governed by the phosphorylation and dephosphorylation of specific tyrosine residues within the cytoplasmic domain of the receptor itself and numerous other cellular proteins involved in the signaling cascade. Upon ligand binding, growth factor receptor tyrosine kinases undergo dimerization of receptor molecules resulting in the activation of their intrinsic tyrosine kinase (Pantoliano et al., 1994; Spivak-Kroizman et al., 1994; Williams, 1989; Ullrich & Schlessinger, 1990; Fantl et al., 1993). Phosphotyrosine residues within the receptor's cytoplasmic domain are generated by either autophosphorylation or transphosphorylation by the other receptor chain in the dimeric complex. The phosphotyrosines can subsequently serve as specific association sites for cellular proteins with domains that have high-affinity binding to specific phosphotyrosine residues of the receptor cytoplasmic domain. Only two cytoplasmic proteins have been shown to physically associate with FGFR1 upon ligand activation, albeit at low levels: PLC-γ, an important mediator of the phosphatidylinositol secondary messenger pathway (Mohammadi et al., 1991), and cortactin (Zhan et al., 1993b). Other signaling molecules such as phosphatidylinositol 3-kinase or GTPase activating protein that become associated with many activated growth factor receptors do not seem to interact at detectable levels with FGFR1 *in vivo* (Peters et al., 1992). In the case of PLC-γ, FGFR1 phosphotyrosine residue 766 has been shown to be responsible for the association (Peters et al., 1992). A point mutation of tyrosine 766 in FGFR1 selectively eliminates tyrosine phosphorylation and activation of PLC-γ; however, neither Ca^{2+} mobilization nor phosphoinositide hydrolysis is required for aFGF-induced mitogenesis. Recent studies suggest that activation of PLC-γ might mediate chemotaxis, cell-shape changes, or cellular differentiation during early embryonic development. It was furthermore found that the point mutation at Y766 resulted in a decrease in FGFR1 internalization, as well as reduction in both ligand-induced FGFR1 downregulation and degradation (Sorokin et al., 1994). Y766 therefore seems to be essential in cellular trafficking of FGFR1.

We have shown that binding of aFGF to the FGFR1′ variant transfected into L6 cells does not lead to receptor auto- or transphosphorylation. Consequently, as shown by others (Mohammadi et al., 1991; Peters et al., 1992), proteins, such as PLC-γ, that normally associate with the wild-type receptor upon ligand binding cannot bind to the cytoplasmic tail of the variant receptor form and do not get activated. As a result, FGFR1′ variant transfectant cell lines are mitogenically inactive when stimulated with aFGF. This makes the FGFR1′ variant a signaling-deficient receptor with presumably dominant negative activities. In order to prove that the FGFR1′ kinase-deficient variant is indeed a dominant negative receptor, we are currently in the process of establishing FGFR1/FGFR1′ double-transfectant L6 cell lines. These transfectant cell lines as well as the above-mentioned human breast cancer cell lines with altered FGFR1/FGFR1′ ratios will allow us to carry out detailed signal transduction studies and address the relevance of the FGFR1′ variant in cellular growth control.

The newly discovered FGFR1 mutant form may be important in the downregulation of aFGF-induced signal transduction events by heterodimerization with full-length FGFR1, leading to a nonfunctional receptor dimer. It is conceivable that in cells expressing both receptor variants the formation of three types of dimeric receptor complexes can take place. After ligand binding FGFR1 and FGFR1′ homodimers and FGFR1/FGFR1′ heterodimers could form. Whereas the FGFR1′ homodimers do not lead to tyrosine auto- or transphosphorylation events due to the kinase deficiency of this variant, the ligand-stimulated heterodimer could result in autophosphorylation of the FGFR1 chain as well as transphosphorylation of the FGFR1′ chain by the active FGFR1. It is therefore conceivable that certain cytoplasmic proteins involved in signaling, such as PLC-γ, can associate with heterodimeric receptor complexes and become activated, but to a smaller extent. Alternatively, the kinase-deficient receptor could also act as a competitive inhibitor for binding of ligand to functional wild-type receptor. Studies to investigate these possibilities and signaling events in FGFR1/FGFR1′ cotransfectant cell lines are in progress. On the basis of the discovery of a novel kinase-deficient FGFR1 variant, we hypothesize that alternative splicing of a growth factor receptor mRNA may be used for cellular growth control. As both forms of the receptor are made by the same cell, it is conceivable that this mechanism is important in normal regulation of cell growth and differentiation and may be impaired in cancerous growth and other proliferative diseases (Folkman & Shing, 1992). Another receptor that is a member of the EGFR family, erbB3, has also been shown to have a greatly reduced tyrosine kinase activity (Carraway & Cantley, 1994; Sliwkowski et al., 1994; Guy et al., 1994). This receptor has changes in four amino acid residues that are highly conserved in the catalytic domain of tyrosine kinases. As a consequence erbB3 undergoes negligible tyrosine autophosphorylation upon ligand binding and it has been proposed to have growth regulatory functions by pairing with other members of the EGFR family (Carraway & Cantley, 1994).

Other studies have implicated the importance of alternatively spliced, albeit structurally unrelated, forms of FGFRs in cardiovascular disease as well as in cancer. In the first study it was shown that the pattern of expression of FGFR1 isoforms generated by alternative splicing was altered significantly during accelerated coronary atherosclerosis in cardiac transplants (Zhao et al., 1994). In another study it was found that different patterns of alternate transcripts of FGFR2 are associated with the induced epithelial−mesenchymal transition in rat bladder carcinoma cells (Savagner et al., 1994).

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