MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 977–989 Vol. 16, No. 3
0270-7306/96/$04.00+0

Identification of Six Novel Autophosphorylation Sites on Fibroblast Growth Factor Receptor 1 and Elucidation of Their Importance in Receptor Activation and Signal Transduction

M. MOHAMMADI,[1] I. DIKIC,[1] A. SOROKIN,[1] W. H. BURGESS,[2] M. JAYE,[3] AND J. SCHLESSINGER[1]*

Department of Pharmacology, New York University Medical Center, New York, New York 10016[1]; American Red Cross, Rockville, Maryland 20855[2]; and Rhone-Poulenc Rorer Central Research, Collegeville, Pennsylvania 19426[3]

Received 28 September 1995/Returned for modification 7 November 1995/Accepted 12 December 1995

Fibroblast growth factor receptor (FGFR) activation leads to receptor autophosphorylation and increased tyrosine phosphorylation of several intracellular proteins. We have previously shown that autophosphorylated tyrosine 766 in FGFR1 serves as a binding site for one of the SH2 domains of phospholipase Cγ and couples FGFR1 to phosphatidylinositol hydrolysis in several cell types. In this report, we describe the identification of six additional autophosphorylation sites (Y-463, Y-583, Y-585, Y-653, Y-654, and Y-730) on FGFR1. We demonstrate that autophosphorylation on tyrosines 653 and 654 is important for activation of tyrosine kinase activity of FGFR1 and is therefore essential for FGFR1-mediated biological responses. In contrast, autophosphorylation of the remaining four tyrosines is dispensable for FGFR1-mediated mitogen-activated protein kinase activation and mitogenic signaling in L-6 cells as well as neuronal differentiation of PC12 cells. Interestingly, both the wild-type and a mutant FGFR1 (FGFR1-4F) are able to phosphorylate Shc and an unidentified Grb2-associated phosphoprotein of 90 kDa (pp90). Binding of the Grb2/Sos complex to phosphorylated Shc and pp90 may therefore be the key link between FGFR1 and the Ras signaling pathway, mitogenesis, and neuronal differentiation.

Fibroblast growth factors (FGFs) constitute a large family of at least nine distinct polypeptide growth factors (7, 31, 34). FGFs play an important role in the regulation of cell growth, differentiation, embryogenesis, and angiogenesis (34). Like other growth factors, FGFs exert their action by binding to and activating a distinct family of growth factor receptors that has been previously classified as subclass IV (20, 74). The FGF receptor family consists of at least four distinct gene products, each composed of an extracellular ligand-binding domain that contains three immunoglobulin-like domains, a single transmembrane domain, and a cytoplasmic domain that contains protein tyrosine kinase activity (interrupted by an insertion of 14 amino acids in the kinase domain). One of the characteristic features of the FGF receptor family is the occurrence of numerous receptor isoforms that are produced from alternatively spliced transcripts in both the intracellular and extracellular domains (9, 14, 27, 32, 33). As with other growth factors, binding of FGF to FGF receptors leads to receptor dimerization (3, 70, 73) and subsequent tyrosine autophosphorylation and phosphorylation of target substrates (6, 13, 23). Autophosphorylation on tyrosine is considered to have at least two functions. One such function is the stimulation of the intrinsic protein tyrosine kinase activity by an allosteric mechanism, as seen with the insulin receptor (22, 63, 81, 84, 86). Also, many autophosphorylation sites serve as binding sites for signaling proteins that contain Src homology 2 (SH2) domains or the recently identified phosphotyrosine interaction (also called phosphotyrosine-binding [PTB]) domains (4, 5, 8, 35, 36, 39, 48, 56, 57). Binding of SH2 or PTB domain-containing proteins to activated growth factor receptors has been shown to be important for the activation of downstream signaling molecules. For example, binding of Shc and phospholipase Cγ (PLCγ) through PTB and SH2 domains, respectively, to the activated nerve growth factor receptor (Trk) has been shown to be required for the nerve growth factor-induced activation of Ras signaling pathways and neuronal differentiation of PC12 cells (16, 55, 72). Several studies have demonstrated that mutation of autophosphorylation sites in platelet-derived growth factor (PDGF) receptor and in colony-stimulating factor 1 receptor can impair mitogenic signaling in some cell lines (19, 76, 77).

Very little is known about the cellular substrates and target proteins involved in signaling processes that lead to FGF-mediated mitogenesis. So far only PLCγ has been shown to associate with the activated FGF receptor 1 (FGFR1) (*flg*); the identities of other targets remain unclear. We have previously identified tyrosine 766 of FGFR1 as the major autophosphorylation site and have shown that this tyrosine and its flanking sequences represent a high affinity binding site for one of the SH2 domains of PLCγ (52). Mutation of this tyrosine to phenylalanine results in a receptor that is no longer able to stimulate phosphatidylinositol (PI) hydrolysis but that can still induce mitogenesis in L-6 myoblasts and BaF3 cells and neurite outgrowth in PC12 cells. Moreover, PI hydrolysis seems to be dispensable for the induction of chemotaxis by acidic FGF (aFGF) in certain cell types (11). These observations indicate that activation of target proteins crucial for either mitogenesis, differentiation, or chemotaxis is not dependent on autophosphorylated tyrosine 766 (29, 51, 58, 71). However, autophosphorylation on tyrosine 766 of FGFR1 is required for efficient endocytosis of FGF receptors (69).

As part of our effort to understand FGFR1 signal transduction, we have mapped six additional autophosphorylation sites (Y-463, Y-583, Y-585, Y-653, Y-654, and Y-730) on FGFR1 and have studied their roles in various FGFR1-mediated responses. Two of the identified tyrosines (Y-653 and Y-654) are

* Corresponding author. Mailing address: Department of Pharmacology, New York University Medical Center, 550 First Ave., New York, NY 10016. Phone: (212) 263-7122. Fax: (212) 263-7133.

located in the kinase domain in the region homologous to tyrosine 416 of pp60$^{c\text{-}src}$. Mutation of these tyrosines reveals that their autophosphorylation is essential for activation of FGFR1. Tyrosines Y-653 and Y-654 are conserved among all known FGF receptors, and we suggest that homologous regions in other FGF receptors are also important in receptor activation. We also generated mutated FGF receptors in which the remaining four autophosphorylation sites (Y-463, Y-583, Y-585, and Y-730) together (FGFR1-4F) or in combination with the previously identified tyrosine 766 (FGFR1-5F) were mutated to phenylalanine. Our results indicate that autophosphorylation of these tyrosines is dispensable for FGF-mediated cell proliferation in L-6 myoblasts as well as neuronal differentiation in PC12 cells. We have found that the FGFR1-5F is still able to phosphorylate Shc and an as yet unidentified, Grb2-associated phosphoprotein with a molecular mass of 90 kDa (pp90).

MATERIALS AND METHODS

Site-directed mutagenesis and generation of stable cell lines. Site-directed mutagenesis was performed according to the protocol of the manufacturer (Amersham). The cDNAs encoding the full-length human wild-type FGFR1 (*flg*) and the FGFR1 mutant with a Y-to-F mutation at position 766 (Y766F mutant) were subcloned into the m13MP19 replicative form with *Bam*HI-*Hin*dIII cloning sites (51). Point mutations were introduced with the oligonucleotides 5′-CACATC GACTTCTATAAAAAGACA-3′ (for the Y653F mutant), 5′-CACATCGACT ACTTTAAAAAGACA-3′ (for the Y654F mutant), and 5′-CACATCGACTTC TTTAAAAAGACA-3′ (for the [Y653/654F mutant), 5′-GTCTCTGAGTTT GAGCTTCCC-3′ (for the Y463F mutant), 5′-CCAGGGCTGGAATTCTGCT TCAACCCCAGCCAC-3′ (for the Y583/585F mutant), and 5′-ACCAAC GAGCTGTTCATGATGATGCGG-3′ (for the Y730F), mutant). The triple-mutant FGF receptor Y463/583/585F was made on the double mutant Y583/585F as the template. The Y463/583/585F triple mutant ssm13 was then used to generate the Y463/583/585/730F (FGFR1-4F) mutant FGF receptor. The Y583/585/766F triple-mutant receptor was generated on the mutant Y766F as the template. The triple mutant Y583/585/766F was then used to generate the Y463/583/585/766F mutant receptor. The Y463/583/585/766F ssm13 was used as the template to generate the Y463/583/585/730/766F (FGFR1-5F) mutant FGF receptor. The different mutated m13MP19 constructs were digested with *Bam*HI and *Hin*dIII, blunted with Klenow enzyme, and subcloned into pMJ30 under the control of adenovirus major late promoter and cytomegalovirus enhancer. L-6 myoblasts, which lack endogenous FGF receptors, were transfected with 0.5 μg of pSV2neo and 40 μg of wild-type or mutant FGFR1 in pMJ30-expressing vectors by calcium phosphate precipitation (10). Clones were isolated after 2 to 3 weeks of selection in G418 (Gibco) and screened for expression of FGFR1 by binding assay with ^{125}I-labeled aFGF as a specific probe. Cell lines expressing similar levels of FGF receptors were used for further analysis.

In addition, cDNAs encoding FGFR1-Y653F, FGFR1-Y654F, FGFR1-Y653/654F, FGFR1-4F (Y463/583/585/730F), and FGFR1-5F (Y463/583/585/730/766F) were subcloned in pRK5 mammalian expression vector with the *Bam*HI and *Hin*dIII cloning sites. PC12 cells were cotransfected with 0.5 μg of pSV2neo and 20 μg of mutant FGFR1 with a Bio-Rad electroporator set at 250 V and 960 μF. Cells were grown for 4 to 5 weeks in medium containing G418, and stable cell lines expressing approximately equal numbers of mutant receptors per cell were isolated by a binding assay with ^{125}I-labeled aFGF and/or immunoblotting with anti-FGFR1 antibodies. The construction of the deletion mutant FlgΔCOOH-FGFR1 and generation of L-6 myoblasts overexpressing this mutant receptor have been described previously (69). FlgΔCOOH-FGFR1 lacks the 56 carboxy-terminal amino acids of the receptor; therefore, tyrosine 766 is not present in this receptor.

Expression of the FGFR cytoplasmic domain in insect cells. The cDNA construct encoding the cytoplasmic domain of FGFR1 was subcloned into baculovirus transfer vector pBlueBac HistagB with *Nco*I and *Hin*dIII cloning sites (Invitrogen). This construct lacks the 56 carboxy-terminal amino acids of the cytoplasmic domain, since the codon for tyrosine (TAC) was changed to a stop codon (TAG) by site-directed mutagenesis. Therefore, tyrosine 766 is not present in this construct. Transfection of insect cells (Sf9) was performed with the BaculoGold transfection system according to the protocol of the manufacturer (Pharmingen). Following identification of the positive plaques, the recombinant virus was amplified to high titer (multiplicity of infection, 10^8). The fusion protein was first purified over an Ni^{2+}-chelating column and then analyzed by anion exchange chromatography (Mono Q column). The fractions containing the fusion protein were concentrated with Centricon 10 (Amicon), and the histidine tag was removed by digestion with enterokinase (Biozyme) overnight. The cleaved kinase domain was then separated from the histidine tag by size exclusion (Superose 12 column) and an additional round of anion exchange (Mono Q column) chromatography. The purity of the kinase was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and was greater than 95%.

In vivo phosphorylation of FGFR1. L-6 myoblasts expressing wild-type FGFR1 or the deletion mutant FlgΔCOOH were starved overnight in Dulbecco's modified Eagle medium (DMEM) containing 0.1% fetal bovine serum (FBS) and then were prelabeled with $^{32}P_i$ (1 mCi/ml) for 2 h in phosphate-free medium (Gibco). Following stimulation with aFGF (100 ng/ml; 5 min at 25°C), the cells were washed with phosphate-buffered saline (PBS) on ice and lysed in lysis buffer containing phosphatase inhibitors. The lysates were spun at 13,000 × *g* in a microcentrifuge for 10 min at 4°C, and the supernatants were immunoprecipitated with anti-FGFR1 antibodies (anti-Flg3B) for 2 h at 4°C. The anti-Flg3B antibodies were raised against a peptide derived from the kinase insert region of FGFR1 (residues 580 to 596) and were used for immunoprecipitation of both the wild type and the deletion mutant. The immunoprecipitates were washed several times with lysis buffer, 3× sample buffer was added, the solution was boiled for 5 min, and the products were analyzed by SDS-PAGE (7% polyacrylamide). The gel was dried, and the ^{32}P-labeled bands corresponding to the FGFR1 were cut out and digested with trypsin as described previously (52). The eluted ^{32}P-labeled peptides were filtered, soybean trypsin inhibitor (10 μg/ml) was added, and the solution was incubated with monoclonal anti-phosphotyrosine antibodies immobilized on beads (Oncogene Science) for 2 h at 4°C. The beads were washed three times with washing buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 50 mM NaCl). The phosphotyrosine-containing peptides were then eluted with 1 ml of 50 mM phenylphosphate in washing buffer, filtered, and directly resolved on an Aquapore C_{18} reverse-phase high-performance liquid chromatography (HPLC) column (4.6 by 250 mm) at a flow rate of 1 ml/min with an acetonitrile gradient in 0.1% aqueous trifluoroacetic acid. The following gradient was used: 0 to 10 min, 0% CH_3CN; 10 to 70 min, linear gradient to 60% CH_3CN. Fractions of 0.5 ml were collected, and ^{32}P radioactivity was analyzed with a beta counter.

Identification of tyrosine autophosphorylation sites of FGF receptor. The purified recombinant kinase domain (200 μg) was subjected to an in vitro kinase assay in the presence of 10 mM Mg^{2+}, 5 mM ATP, and a trace amount of [γ-^{32}P]ATP (10 μCi) for 5 min at room temperature. We used a trace amount of [γ-^{32}P]ATP in order to monitor the tyrosine-phosphorylated peptides during tryptic digestion and reverse-phase HPLC analysis. The ^{32}P-labeled autophosphorylated kinase domain was analyzed by SDS-PAGE (12% polyacrylamide) in order to remove the unincorporated [γ-^{32}P]ATP. The gel was dried, and the radioactive bands corresponding to the kinase domain were excised from the gel and subjected to tryptic digestion as described previously (52). The eluted peptides were then filtered, and soybean trypsin inhibitor (10 μg/ml) was added. The mixture was then incubated with monoclonal anti-phosphotyrosine antibodies immobilized on beads (Oncogene Science) for 2 h at 4°C. The beads were washed three times with washing buffer, and the phosphotyrosine-containing peptides were eluted with phenylphosphate (see above). The ^{32}P-labeled peptides were resolved on an Aquapore C_{18} reverse-phase HPLC column with the same gradient as described above. Fractions of 0.5 ml were collected, and ^{32}P-labeled fractions were analyzed by a pulsed liquid-phase protein Microsequencer (model 477A with an on-line model 120 phosphothiohydantoin analyzer; Applied Biosystems Inc., Foster City, Calif.). About 20 to 50 pmol of the ^{32}P-labeled phosphopeptides was used for microsequencing. All residues were correctly identified as phosphothiohydantoin-derived amino acids, except tyrosine residues which were phosphorylated. The inability to detect tyrosine-phosphorylated residues is expected, since the charged phosphate group prevents the extraction of the tyrosyl derivative into the organic phase in the sequencer.

Phosphoamino acid analysis. Following tryptic digestion of the gel pieces containing the ^{32}P-labeled autophosphorylated kinase domain, the eluted phosphopeptides were dried and the residual ammonium bicarbonate was removed by evacuation three times with water and later hydrolyzed in 50 μl of 6 N HCl for 1 h at 110°C. Samples were dried, and the residual HCl was removed by evacuation twice with water. The samples were dissolved in electrophoresis buffer (pH 1.9) containing phosphoamino acid standards and then spotted onto cellulose thin-layer chromatography plates. Phosphoamino acids were resolved by two-dimensional electrophoresis (pH 1.9 followed by pH 3.5) as described previously (12).

Immunoprecipitation and immunoblot analysis. Cells expressing wild-type or mutant receptors were grown in 10-cm-diameter dishes until 80 to 90% confluency was reached. The cells were starved overnight in DMEM containing 0.1% FBS and then treated with aFGF (100 ng/ml) for 5 min at 37°C. The cells were washed briefly with cold PBS, and lysed in 1 ml of lysis buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride) containing phosphatase inhibitors (50 mM sodium fluoride, 10 mM sodium PP_i, and 0.2 mM sodium orthovanadate). Lysates were incubated with appropriate antibodies overnight. Protein A-Sepharose was added, and after 2 h the immunocomplexes were washed three times with HNTG (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). Sample buffer (3×) was added, and the samples were boiled for 5 min. After analysis by SDS-PAGE, proteins were transferred electrophoretically onto nitrocellulose membranes and immunoblotted with different antibodies. Blots were treated with ^{125}I-labeled protein A and analyzed by autoradiography. The following antibodies were used

throughout these studies: anti-FGFR1 (29); anti-PLCγ1 (45); anti-Sos1 (44), and anti-Grb2 and anti-Shc (2). In the in vivo labeling experiments we used an anti-Flg3B antiserum. These antibodies were raised against the kinase insert region of FGFR1 (residues 580 to 596) (52).

In vitro kinase assay. Cells expressing wild-type and FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F mutant receptors were stimulated with aFGF (100 ng/ml) for 5 min at 37°C, lysed, and immunoprecipitated with anti-FGFR1 receptor antibodies. The immunoprecipitates were subjected to an in vitro kinase assay (50 μl) in the presence of [γ-^{32}P]ATP (5 μM), 10 mM Mg_2Cl, and an exogenous substrate, either 5 mM angiotensin or a fusion protein containing the carboxy-terminal tail of FGFR1 fused to glutathione *S*-transferase (GST-FGFR1-CT) (10 μg per reaction), for 5 min. Sample buffer was added, and the reaction products were analyzed by SDS-PAGE and autoradiography. Angiotensin was separated from [γ-^{32}P]ATP by using a gradient gel of 5 to 15% (15 ml) that was layered on the top of a previously poured 20% gel (10 ml).

PI hydrolysis. L-6 myoblasts expressing wild-type or mutant receptors were labeled with [^{3}H]myoinositol (2 μCi/ml) in DMEM containing 0.5% FBS for 24 h and incubated in DMEM containing 20 mM LiCl for 20 min before addition of aFGF (100 ng/ml) for an additional 30 min at 37°C. Cells were extracted with perchloric acid and inositol phosphate formation was measured according to published procedures (49).

[^{3}H]thymidine incorporation. L-6 cells were seeded in 96-well plates (2×10^4 cells per well), and 24 h later the medium was changed to DMEM containing 0.1% FBS. After 48 h of serum starvation, the medium was replaced by medium containing aFGF or, as control, 10% FBS. After stimulation for 24 h, cells were incubated with [^{3}H]thymidine (final concentration, 0.5 μCi/ml) for 16 h at 37°C. Cells were washed with PBS, trypsinized, and collected with a PHD cell harvester (Cambridge Technologies), and the amount of incorporated [^{3}H]thymidine was quantitated by a liquid scintillation counting device (LKB).

PC12 cell differentiation assay. To examine the effect of FGF on morphology of PC12 cells, cells were seeded at a density of 1.5×10^5 cells per 6-cm-diameter tissue culture dish in DMEM supplemented with 3% horse serum, 2.5% fetal calf serum, penicillin, streptomycin, and L-glutamine. Cells were stimulated with aFGF (100 ng/ml) or with medium alone as a control. The kinetics and extent of growth factor-induced neurite outgrowth were analyzed by randomly scoring 500 cells for each plate and calculation of the percentage of cells with neurites longer than two cell bodies as previously described (17).

RESULTS

Identification of autophosphorylation sites in FGFR1. The intracellular domain of FGFR1 was expressed as a histidine-tagged fusion protein in Sf9 cells with a baculovirus-based expression system. The recombinant protein was purified to homogeneity by metal chelating, anion exchange, and size exclusion chromatography. The purified kinase domain was phosphorylated in vitro in the presence of [γ-^{32}P]ATP and $MgCl_2$ and was further analyzed by SDS-PAGE (Fig. 1A). Phosphoamino acid analysis of the in vitro-phosphorylated kinase domain revealed the presence of only phosphotyrosine in the autophosphorylated FGFR1 kinase domain (Fig. 1B). Following tryptic digestion, the phosphopeptides were purified on an anti-phosphotyrosine antibody affinity column and were separated by reverse-phase HPLC. Seven different peaks were detected by HPLC (Fig. 1C) and were further analyzed by microsequencing (Fig. 1D). Peak 1 was shown to be a doubly phosphorylated peptide with phosphate groups on both tyrosines 653 and 654. Peak 2 contained the same peptide as peak 1 except that only tyrosine 653 was found to be phosphorylated. Peak 1 was a partially digested peptide, since trypsin did not cleave on the carboxy-terminal site of the lysine 655, while peak 2 was cleaved at this site. We did not detect any peptide that was phosphorylated on tyrosine 654 alone. Peak 3 represented a phosphopeptide derived from the kinase insert region of the FGF receptor, which contained phosphorylated tyrosines 583 and 585. Peak 4 contained the same peptide as peak 3, except that only tyrosine 583 was phosphorylated. Peak 5 was derived from the juxtamembrane region, where tyrosine 463 was found to be phosphorylated. Peaks 6 and 7 each contained a phosphate group on tyrosine 730, a residue located in the second half of the kinase domain. Since the expressed kinase domain lacks 56 carboxy-terminal amino acids, we did not detect peaks (peaks 8 and 9) corresponding to autophosphorylated tyrosine 766 (Fig. 1C).

Next we compared the tryptic phosphopeptide map of the recombinant kinase domain with the tryptic phosphopeptide maps of wild-type FGFR1 or a deletion mutant (FlgΔCOOH) phosphorylated in living cells. L-6 myoblasts expressing the wild-type or the deletion mutant (FlgΔCOOH) receptors were prelabeled with P_i, treated with aFGF, and lysed, and the lysates were immunoprecipitated with anti-FGFR1 antibodies. Following SDS-PAGE analysis, the ^{32}P-labeled receptor bands were digested with trypsin and the eluted phosphopeptides were purified on an anti-phosphotyrosine affinity column. The mixture of the tyrosine-phosphorylated ^{32}P-labeled phosphopeptides was then resolved by reverse-phase HPLC (Fig. 1E). Seven different ^{32}P-labeled phosphopeptides (peaks 1 through 7) with similar retention times to those of the phosphopeptides obtained from in vitro-phosphorylated recombinant kinase domain were detected in the HPLC maps of both the in vivo-labeled wild-type receptor as well as the deletion mutant FlgΔCOOH FGF receptor. Moreover, one additional phosphopeptide (peak 8) was observed in the HPLC map of the in vivo-labeled wild-type FGF receptor. This phosphopeptide (peak 8) represents the carboxy-terminal autophosphorylation site (tyrosine 766), and as expected this phosphopeptide is absent from the HPLC map of the deletion mutant as well as from the HPLC map of the in vitro-phosphorylated recombinant kinase domain. The phosphorylation of tyrosines 583 and 585, peaks 3 and 4 (derived from the kinase insert region), in vivo was weaker than the phosphorylation of the same sites detected in in vitro-phosphorylated recombinant kinase domain. This could be due to rapid dephosphorylation of these sites by intracellular phosphatases or due to the fact that the antibodies used for immunoprecipitation are directed against the kinase insert region of FGFR1. Phosphorylation of these two tyrosines may affect the ability of the anti-Flg3B antibodies to recognize the kinase insert region of FGFR1. Furthermore, phosphopeptide 1, which contains phosphate groups on both tyrosines 653 and 654, is less abundant in vivo than the phosphopeptide derived from the in vitro-phosphorylated kinase domain. In vitro after 1 min of incubation peak 2 (Tyr-653) is the major phosphorylation site (Fig. 1F). Peak 2 is also a major autophosphorylation site in the HPLC maps of the in vivo-labeled wild type as well as the deletion mutant. We suggest that peak 2 is a doublet because of the presence of two adjacent arginine residues (R-655 and R-656). Trypsin will cleave either after R-656 or between the two arginines, causing a slight change in the hydrophobicity of phosphopeptide 2. We suggest that peak 1 is not a doublet, because it contains a negatively charged phosphate group on tyrosine 654. The presence of a negatively charged phosphate group may affect the hydrolysis of the peptide bond connecting R-655 and R-656. We have shown that tyrosines 766, 653, and 654 are the major tyrosine autophosphorylation sites of FGFR1 in vivo. Determination of the actual stoichiometry of autophosphorylation in vivo is probably an underestimate, since it is impossible to completely prevent the action of tyrosine phosphatases in living cells. Moreover, following in vivo labeling the FGF receptor was immunoprecipitated with anti-FGFR1 antibodies, which may preferentially recognize a certain population of phosphorylated receptors. Furthermore, anti-phosphotyrosine antibodies that were used for isolation of tyrosine-phosphorylated peptides may also select for a certain population of phosphopeptides. Finally, hydrophobic phosphopeptides are usually not well represented in HPLC maps because of their lower levels of recoveries during purification (because of stickiness). For these reasons it is difficult to determine with certainty the

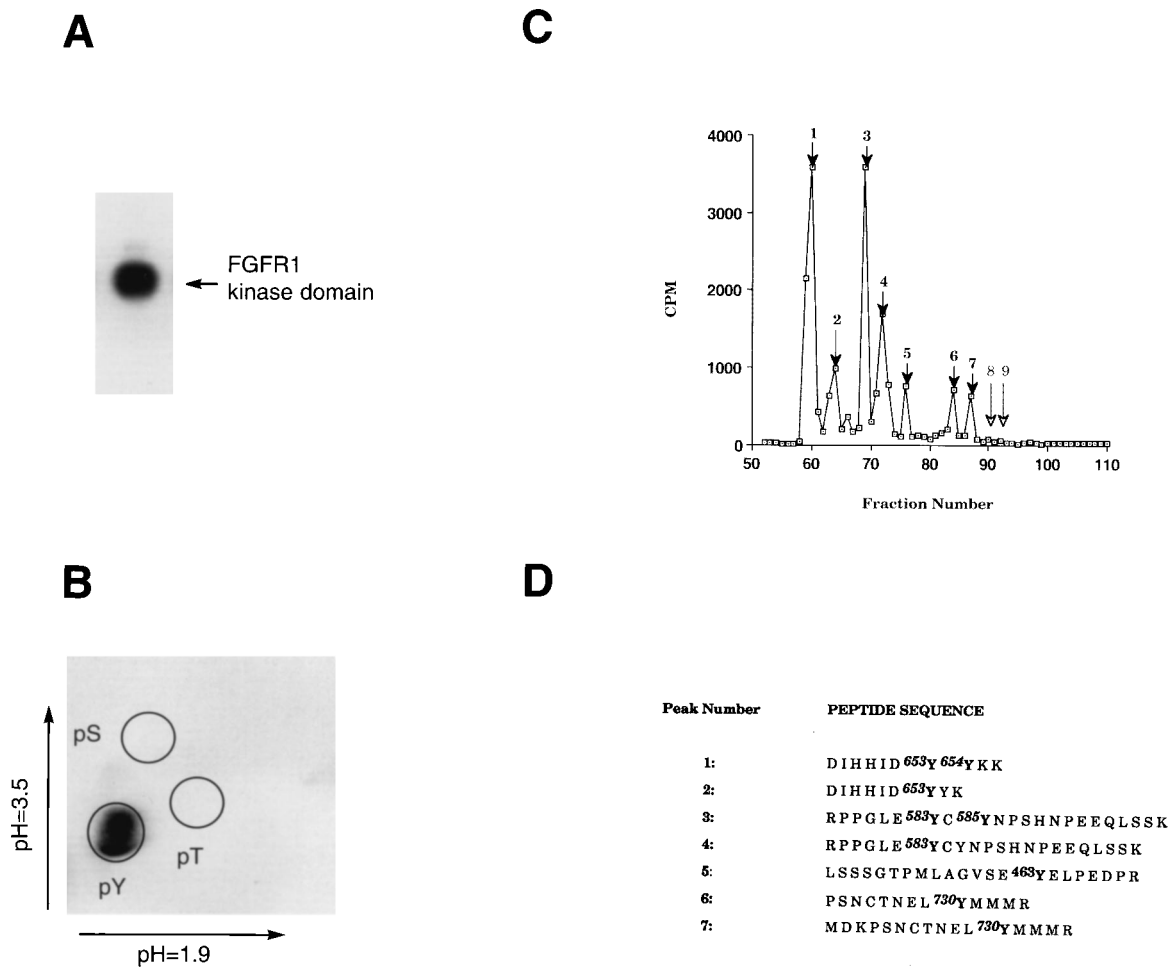

FIG. 1. Identification of novel autophosphorylation sites on FGFR1. (A) In vitro kinase assay of the purified intracellular domain of the FGFR1 expressed in Sf9 cells. The FGFR1 kinase domain was subjected to in vitro autophosphorylation in the presence of [γ-^{32}P]ATP and Mg^{2+}. The reaction product was analyzed by SDS-PAGE and autoradiography. (B) Phosphoamino acid analysis of the in vitro-phosphorylated kinase domain. The autophosphorylated kinase domain band was excised from the gel and subjected to tryptic digestion. The tryptic phosphopeptides were then hydrolyzed and analyzed by two dimensional chromatography. (C) Phosphopeptide map of the FGFR1 cytoplasmic domain analyzed by reverse-phase HPLC. The autophosphorylated FGFR1 kinase domain was digested with trypsin, and phosphotyrosine-containing phosphopeptides were first purified on an anti-phosphotyrosine affinity column and later analyzed by reverse-phase HPLC as described in Materials and Methods. Radioactive peaks numbered from 1 to 7 are shown by solid arrows. The expected positions of phosphopeptides corresponding to tyrosine 766 are indicated by open arrows (peaks 8 and 9). (D) Amino acid sequences of the phosphopeptides analyzed by microsequencing. The purified phosphopeptides isolated from HPLC were sequenced by a pulsed liquid-phase protein Microsequencer as described in Materials and Methods. Note that in peak 6 trypsin cleaves after arginine 721, even though this arginine is followed by a proline. It was shown that trypsin can catalyze the hydrolysis of lysyl and arginyl peptide bonds followed by proline residues, although the rate of hydrolysis may be slower (1). (E) Comparison of the tryptic phosphopeptide map of recombinant kinase domain with the tryptic phosphopeptide maps of wild-type (WT) FGFR1 and the deletion mutant FlgΔCOOH. L-6 myoblasts expressing the wild-type FGFR1 or the deletion mutant FlgΔCOOH were prelabeled with $^{32}P_i$ (1 mCi/ml) for 2 h and stimulated with aFGF (100 ng/ml; 5 min at 25°C). Following cell lysis, the lysates were immunoprecipitated with anti-FGFR1 antiserum and analyzed by SDS-PAGE. The ^{32}P-labeled bands corresponding to FGFR1 were subjected to tryptic digestion, and the phosphotyrosine-containing peptides were purified on an anti-phosphotyrosine affinity column and further resolved by reverse-phase HPLC. Radioactive peaks are numbered 1 through 8. Peak 8 corresponds to the phosphopeptide containing the autophosphorylated tyrosine 766. This peak is absent both from the HPLC maps of the in vitro-phosphorylated kinase domain and from the in vivo-labeled deletion mutant FlgΔCOOH. Peak 2 in the HPLC map of wild-type FGFR1 (in vivo) is split because of partial digestion at the carboxy terminus of arginine 656. (F) Time course of in vitro autophosphorylation of the recombinant kinase domain. The purified recombinant kinase domain was subjected to in vitro autophosphorylation as a function of time (1, 2, and 5 min). Following SDS-PAGE, the ^{32}P-labeled bands corresponding to the autophosphorylated kinase domain were excised from the gel and subjected to tryptic digestion. The eluted ^{32}P-labeled phosphopeptides were resolved by reverse-phase HPLC as described in Materials and Methods. Peaks are numbered 1 through 7. The minor peak observed between peak 2 and peak 3 is due to partial cleavage at the carboxy-terminal site of arginine 656.

exact stoichiometry of phosphorylation of tyrosine phosphorylation sites in living cells.

We have analyzed the tyrosine phosphorylation of the catalytic kinase domain as a function of time and found that tyrosine autophosphorylation is an ordered event. Tyrosine 653 is autophosphorylated prior to tyrosine 654, and autophosphorylation on tyrosine 583 occurs prior to phosphorylation on tyrosine 585 (Fig. 1F). It is possible that efficient autophosphorylation on tyrosines 654 and 585 requires an already-phosphorylated tyrosine residue amino terminal to tyrosines 654 and 585. This notion is consistent with the observation that monophosphorylated peptides with a phosphate group on tyrosine 654 or 585 alone have not been found (Fig. 1F).

Autophosphorylation of tyrosines 653 and 654 is essential for kinase activity of FGFR1 in vitro and in vivo. Two of the newly identified tyrosine autophosphorylation sites are located in a region homologous to tyrosine 416 of pp60$^{c\text{-}src}$. It has been demonstrated in the case of the insulin receptor, the scatter

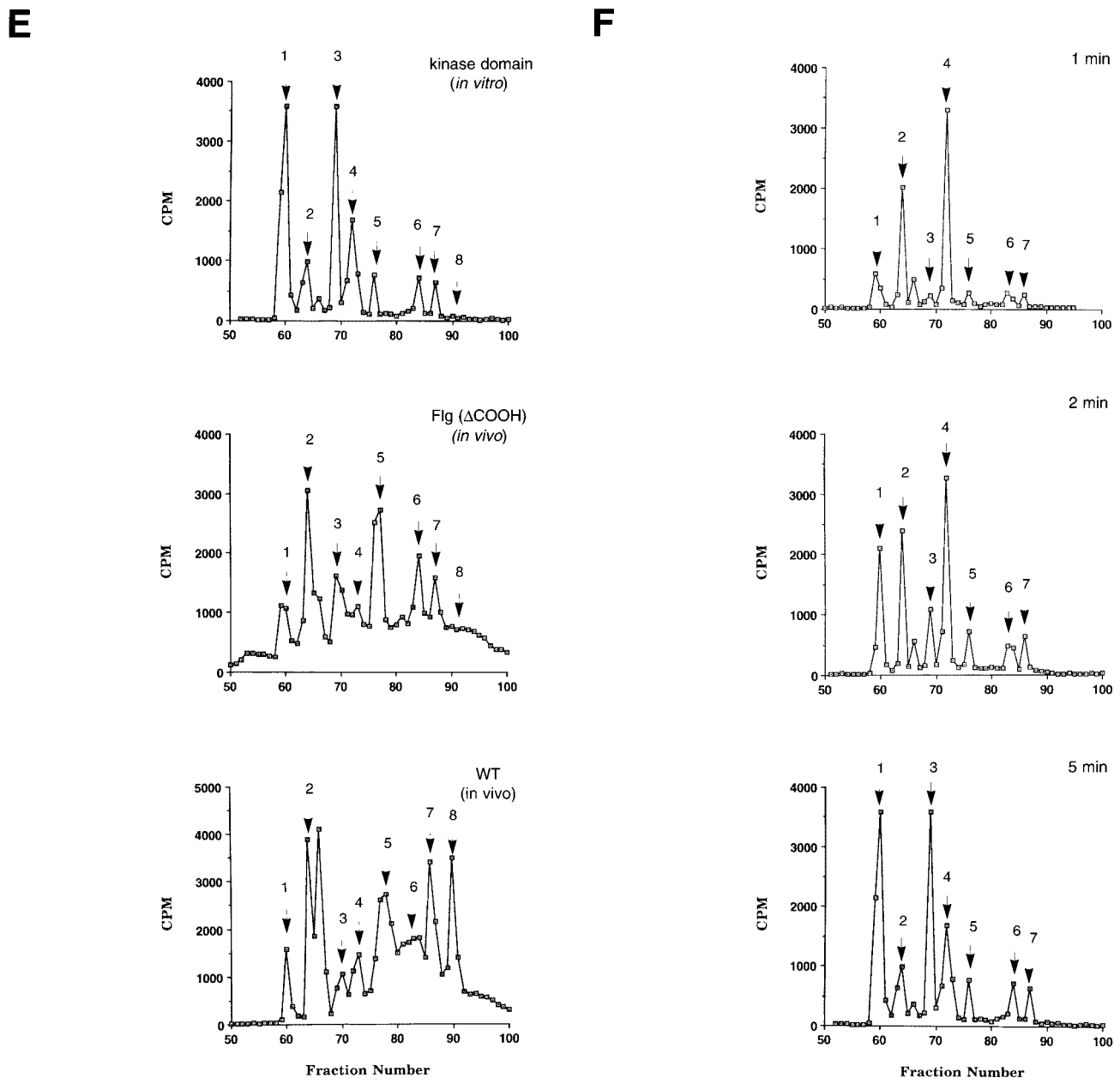

FIG. 1—*Continued.*

factor/hepatocyte growth factor receptor (Met), and the nerve growth factor receptor (Trk) that autophosphorylation of the corresponding tyrosines is required for activation of the catalytic domain (18, 21, 22, 24, 40, 41, 46, 50, 54, 62, 72, 81, 86). We therefore investigated the roles of these two tyrosines in activation of FGFR1 kinase activity in vivo. The singly and doubly mutated receptors FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F were constructed. Wild-type FGFR1 and the mutated receptors FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F were expressed in L-6 myoblasts, which lack endogenous FGF receptors. Several cell lines were generated and characterized. These cell lines were treated with aFGF, lysed, and immunoprecipitated with anti-FGF receptor antibodies, and this was followed by immunoblotting with either anti-FGF receptor (Fig. 2A) or anti-phosphotyrosine antibodies (Fig. 2A). In parallel, lysates from aFGF-stimulated or unstimulated cells were analyzed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 2B). All receptors except the FGFR1-Y653/654F mutant underwent tyrosine autophosphorylation in vivo. The slight increase in the migration rate of the FGFR1-Y654F (Fig. 2A; upper part) is probably due to gel handling for immunoblotting and is not a reproducible finding. While tyrosine phosphorylation of intracellular substrates was comparable with that of wild-type receptor in aFGF-treated cells expressing FGFR1-Y653F, it was diminished in cells expressing FGFR1-Y654F and was completely abolished in cells expressing FGFR1-Y653/654F (Fig. 2B). These data strongly suggest that autophosphorylation of both tyrosines 653 and 654 is required for activation of the kinase domain in vivo. To confirm this point, we compared the abilities of the wild-type

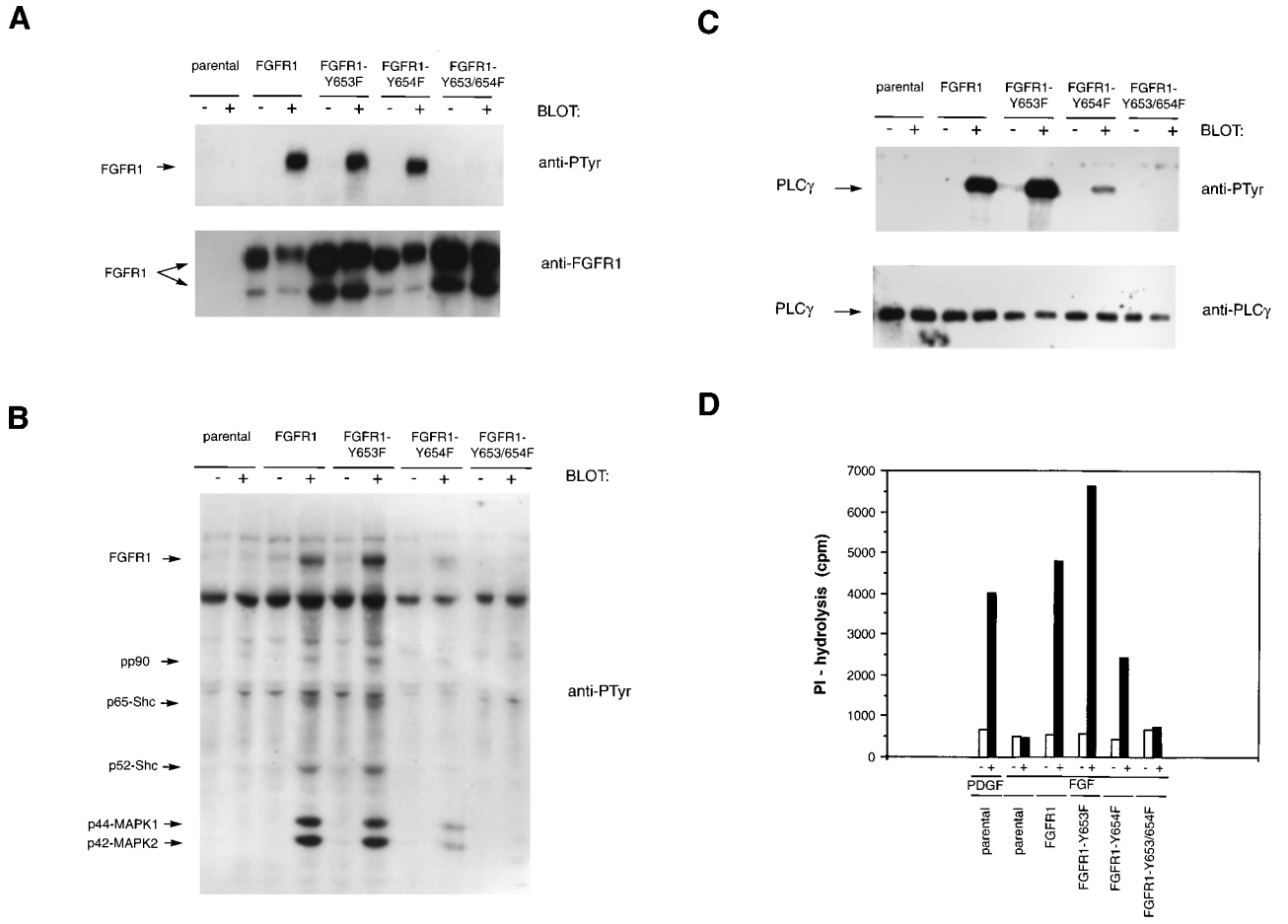

FIG. 2. Mutation of tyrosines 653 and 654 abolishes kinase activity of FGF receptor in vivo. (A) Anti-FGFR1 immunoprecipitates from aFGF-stimulated (+) or unstimulated (−) cells expressing wild-type FGFR1 and mutant receptors FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F were analyzed by immunoblotting with either anti-FGFR1 or anti-phosphotyrosine (anti-PTyr) antibodies. (B) Comparison of tyrosine phosphorylation of intracellular proteins induced by aFGF in cell lines expressing the mutant FGFR1. Lysates from aFGF-treated or untreated cells expressing wild-type FGFR1 or FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F mutant receptors were analyzed by immunoblotting with anti-PTyr antibodies. MAPK1 and MAPK2, MAP kinase 1 and 2, respectively. (C) Tyrosine phosphorylation of PLCγ by wild-type and mutant FGF receptors. Lysates from aFGF-treated or untreated cells expressing wild-type FGFR1 or FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F mutants were immunoprecipitated with anti-PLCγ antibodies and analyzed by immunoblotting with either anti-PLCγ or anti-PTyr antibodies. (D) Analysis of PI hydrolysis induced by wild-type and mutant FGFR1. Cells expressing wild-type FGFR1 or FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F mutant receptors were labeled with [^{3}H]myoinositol for 24 h and then stimulated with aFGF for 30 min. As a control, parental cells were stimulated with PDGF (50 ng/ml) for 30 min. The amount of inositol phosphates was measured as described in Materials and Methods.

and mutant FGFR1s to phosphorylate PLCγ, the best-characterized substrate of FGFR1. Upon treatment of the cells with aFGF, all of the receptors except the Y653/654F mutant were able to phosphorylate PLCγ (Fig. 2C); however, the extent of tyrosine phosphorylation of PLCγ was weak in cells expressing FGFR1-Y654F mutant receptors. As anticipated, we detected no increase in PI hydrolysis in response to aFGF in cells expressing the Y653/654F double-mutant FGF receptors and only a moderate increase in PI hydrolysis in cells expressing the FGFR1-Y654F receptors (Fig. 2D). These experiments demonstrate that mutation of both tyrosines abolishes the ability of the receptor to undergo autophosphorylation and to phosphorylate intracellular substrates in the context of living cells.

We next compared the in vitro kinase activities of the wild-type and mutant FGFR1. Immunoprecipitated receptors were subjected to an in vitro kinase assay in the absence (Fig. 3A) or presence (Fig. 3B and C) of exogenous substrates. As exogenous substrates we used either a fusion protein containing the carboxy-terminal tail of FGFR1 (GST-FGFR1-CT) or angiotensin. Immunoprecipitates from aFGF-treated cells have three- to fivefold higher levels of kinase activity than do immunoprecipitates from untreated control cells (Fig. 3). While the FGFR1-Y653F mutant had in vitro kinase activity comparable to that of the wild-type receptor, the FGFR1-Y654F mutant exhibited a diminished ability to phosphorylate exogenous substrates. Mutation of both tyrosine residues completely prevented the activation of the kinase domain in response to aFGF stimulation. Thus, it appears that autophosphorylation on tyrosines 653 and 654 is required for aFGF-dependent stimulation of kinase activity in both in vitro and in living cells.

We next measured DNA synthesis in cells expressing wild-type and mutated forms of FGFR1 (Fig. 4A and B). Mutation of tyrosine 653 alone did not affect the mitogenic response to aFGF, while the Y654F mutant receptor had a severely reduced ability to stimulate thymidine incorporation. Mutation of both tyrosines abolished the mitogenic response to aFGF completely. The small decrease in thymidine incorporation (less than 50%) in cells expressing FGFR1-Y653F was not a consistent finding, while DNA synthesis in cell lines expressing FGFR1-Y654F and FGFR1-Y653/654F was severely dimin-

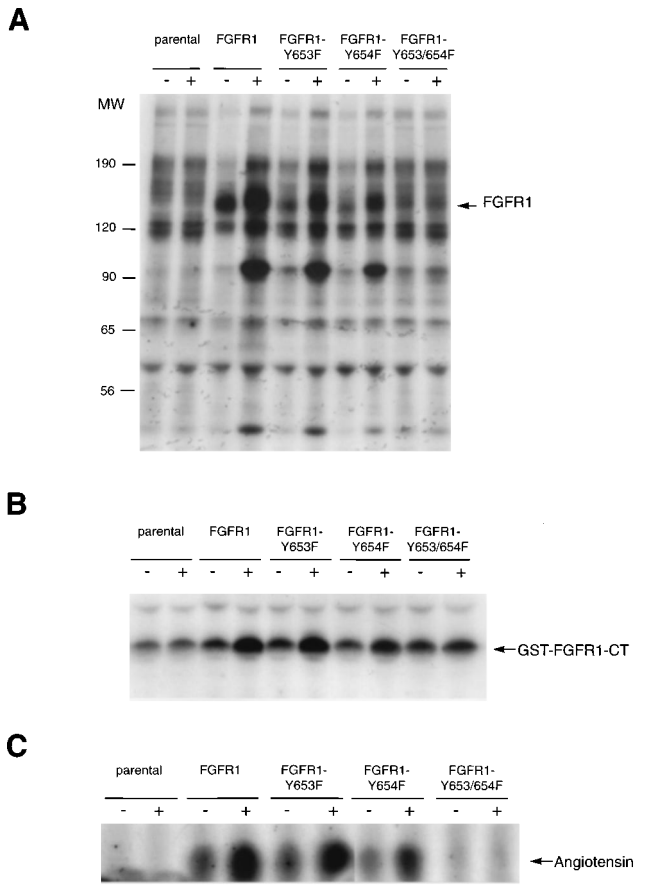

FIG. 3. Autophosphorylation on tyrosines 653 and 654 is required for stimulation of kinase activity of the FGF receptor in vitro. Cells expressing wild-type FGFR1 and the FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F mutant receptors were stimulated with aFGF (+) or left unstimulated (−). Following cell lysis, lysates were immunoprecipitated with anti-FGFR1 antibodies and subjected to in vitro kinase assay in presence of [γ-^{32}P]ATP and Mg^{2+} without (A) or with an exogenous substrate (GST-FGFR1-CT [B] or angiotensin [C]). The reaction products were then analyzed by SDS-PAGE and autoradiography. MW, molecular weight (in thousands).

ished in all the cell lines tested. In addition, we have established stable PC12 cell lines expressing similar levels of wild-type FGFR1 or the three mutant receptors FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F. PC12 cells expressing FGFR1-Y654F had a delayed and less prominent aFGF-induced neurite outgrowth than did wild-type FGFR1 or mutant FGFR1-Y653F (Fig. 4C). FGF stimulation of PC12 cells overexpressing FGFR1-Y653/654F did not induce neuronal differentiation (Fig. 4C). These experiments demonstrated that autophosphorylation of Y-653 and Y-654 is essential for the kinase activity of FGFR1 and hence for mitogenesis in L-6 myoblasts as well as neuronal differentiation in PC12 cells.

Autophosphorylation of four tyrosines in the FGFR1 is not necessary for the activation of mitogen-activated protein (MAP) kinase, mitogenesis, and neuronal differentiation. Autophosphorylation of growth factor receptors on multiple sites generates specific binding sites for a variety of intracellular proteins containing SH2 or PTB domains. It is believed that autophosphorylation sites have a key role in the interaction between activated tyrosine kinase receptors and downstream components in the signal transduction pathways. It has been demonstrated that specificity exists in the interaction between

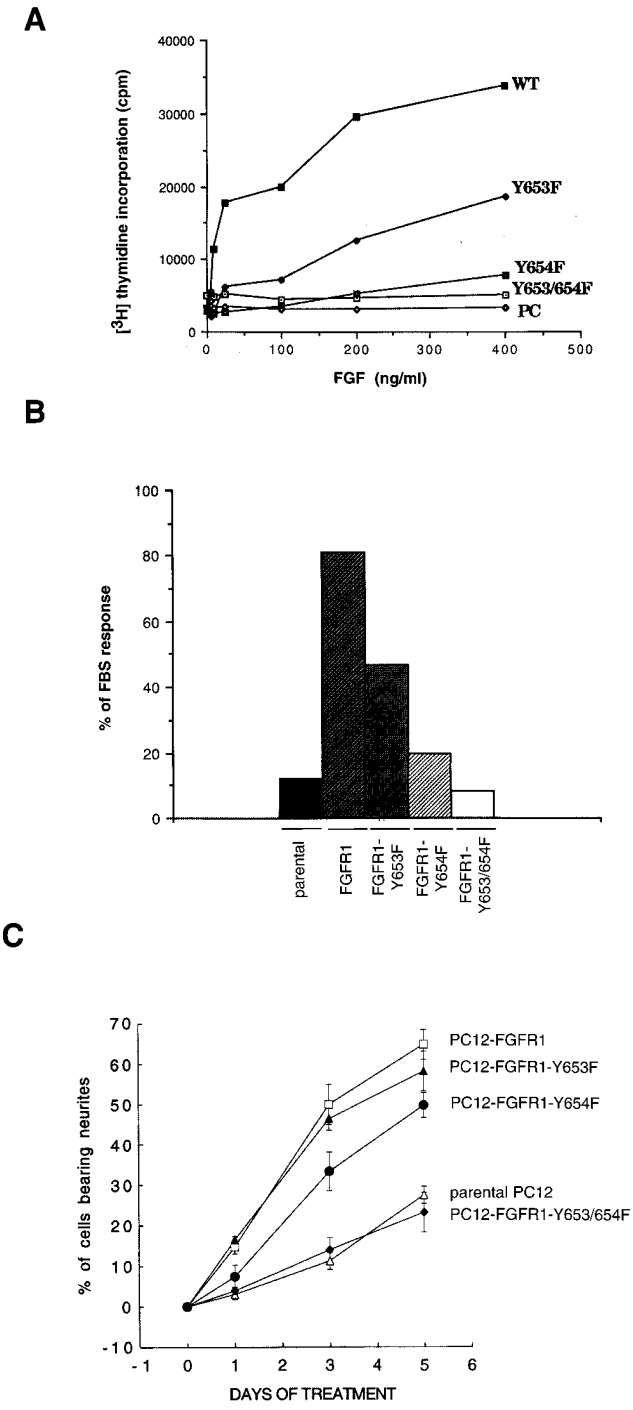

FIG. 4. Autophosphorylation on tyrosines 653 and 654 is necessary for cell proliferation in L-6 myoblasts and neuronal differentiation in PC12 cells. (A) DNA synthesis after aFGF stimulation in cells expressing wild-type (WT) and mutant FGFR1. Stable cell lines expressing wild-type FGFR1 or FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F mutant receptors were starved for 48 h in the presence of 0.1% FBS and then treated with different concentrations of aFGF or, as control, with 10% FBS for 24 h. [^{3}H]thymidine was added, and after an additional 16 h the amount of incorporated [^{3}H]thymidine was quantitated. PC, parental PC12. (B) The results obtained in panel A are expressed as percentage responses from the treatment with 10% FBS. (C) Quantification of aFGF-induced neuronal differentiation of PC12 cells expressing wild-type FGFR1 or FGFR1-Y653F, FGFR1-Y654F, and FGFR1-Y653/654F mutant receptors. Cells were stimulated with aFGF (100 ng/ml) for 5 days, and neurite appearance was quantified by scoring 500 cells from randomly selected light microscope fields of tissue culture dishes. The datum points represent means of three independent experiments (bars, standard errors).

these components and the different autophosphorylation sites in cytoplasmic domains of activated receptor tyrosine kinases (57). Elimination of certain tyrosine phosphorylation sites therefore prevents activation of particular signaling pathways. For example, FGF-induced PI hydrolysis is totally eliminated by a point mutation of FGF receptor at Y-766, which prevents the association with and tyrosine phosphorylation of PLCγ.

It is thought that the activity of a given receptor tyrosine kinase equals the sum of the activities of the signaling molecules that are activated by a given receptor tyrosine kinase (64). Furthermore, it seems that receptor tyrosine kinases utilize several parallel pathways in order to achieve their biological effects. Since the single mutant FGFR-Y766F is still able to induce mitogenesis (51, 58), we examined the possibility that the four autophosphorylated tyrosines (463, 583, 585, and 730) may couple FGFR1 to downstream signaling pathways that lead to mitogenesis. Therefore, we generated mutated forms of FGFR1 in which either four or five of the known autophosphorylation sites were replaced by phenylalanine (FGFR1-4F and -5F, respectively). These mutated receptors were transfected into L-6 myoblasts as well as into PC12 cells, and several stable cell lines were established. Cell lines expressing wild-type or mutant (FGFR1-4F and FGFR1-5F) receptors were treated with aFGF, lysed, and immunoprecipitated with anti-FGF receptor antibodies. The immunoprecipitates were then analyzed by immunoblotting with either anti-FGF receptor (Fig. 5A) or anti-phosphotyrosine antibodies (Fig. 5B). As shown in Fig. 5B, both mutated receptors were able to undergo tyrosine autophosphorylation, albeit to a lesser extent than the wild-type receptor, since four or five of the autophosphorylation sites have been removed. We next compared the pattern of total cellular tyrosine-phosphorylated proteins upon stimulation of wild-type or mutant FGFR1 with aFGF. As shown in Fig. 5C, in both wild-type and mutant cell lines the most prominent tyrosine-phosphorylated bands were MAP kinases, with apparent molecular masses of 42 and 44 kDa (Fig. 5C). In addition, more slowly migrating forms of MAP kinases were noticed in all cells by immunoblotting with anti-Erk-2 antibodies (Fig. 5D). Both experiments showed that mutated receptors were able to activate MAP kinases to extents similar to that of the wild-type FGFR1 (Fig. 5C and D).

The adaptor protein Shc was shown to be tyrosine phosphorylated in response to aFGF stimulation of cells expressing wild-type FGFR1 or the mutant FGFR1-Y766F (71, 75, 78). In order to determine whether tyrosine phosphorylation of Shc requires tyrosine autophosphorylation of a specific site on the FGFR1, L-6 cells expressing wild-type FGFR1 or mutant receptors (FGFR1-4F and FGFR1-5F) were treated with aFGF, lysed, immunoprecipitated with anti-Shc antibodies, and immunoblotted with anti-phosphotyrosine antibodies (Fig. 6A). This experiment showed that mutant receptors were able to tyrosine phosphorylate Shc proteins to the same level as the wild-type receptor. We could not detect tyrosine-phosphorylated FGFR1 in immunoprecipitates of Shc nor vice versa (Fig. 6A and data not shown). We next examined whether the Grb2/Sos complex associates directly or indirectly with the activated wild-type or mutant FGFR1. Lysates from aFGF-treated or untreated cells expressing wild-type or mutant FGFR1 were immunoprecipitated with anti-Grb2 (Fig. 6B) or anti-Sos (Fig. 6C and D) antibodies and were immunoblotted with anti-phosphotyrosine (Fig. 6B and D) or anti-Sos (Fig. 6C) antibodies. Several phosphotyrosine-containing bands, with apparent molecular masses of 90, 65, and 52 kDa, were detected in the anti-Grb2 immunoprecipitates in all of the cell lines tested (Fig. 6B). The 65- and 52-kDa bands represent different forms of Shc, while the identity of the tyrosine-phosphorylated protein with a molecular mass of 90 kDa (pp90) is unknown. Stimulation of cells expressing the FGFR1 mutants caused a mobility shift of Sos to a similar extent as that detected in cells expressing the wild-type FGF receptor (Fig. 6C). Interestingly, we also noticed the presence of the 90-kDa phosphoprotein in the anti-Sos immunoprecipitates from aFGF treated cell lines (Fig. 6D). These results indicate that wild-type and mutant FGFR1 are able to induce complex formation between Grb2/Sos and tyrosine-phosphorylated Shc as well as a yet unidentified tyrosine-phosphorylated protein of 90 kDa.

Finally, we investigated the ability of the wild-type and the mutant FGFR1 to induce DNA synthesis in L-6 cells and neuronal differentiation in PC12 cells. Both the FGFR1-4F and FGFR1-5F mutants were able to induce DNA synthesis with an aFGF dose dependence similar to the dose response obtained with wild-type FGFR1 (Fig. 7A). Furthermore, aFGF induced both accelerated appearance and greater length of neurites in PC12 cells overexpressing the FGFR1-4F and FGFR1-5F mutants to an extent similar to that in PC12 cells overexpressing wild-type FGFR1. These results suggest that the mutated receptors have a capacity equal to that of the wild-type FGFR1 to induce neuronal differentiation (Fig. 7B). It therefore appears that autophosphorylation of none of the five tyrosines (Y-463, Y-583, Y-585, Y-730, and Y-766) is required for activation of mitogenic or differentiation pathways by FGFR1.

DISCUSSION

In this report we describe the identification of new tyrosine autophosphorylation sites in FGFR1 and delineate their roles in receptor activation and signal transduction. The major autophosphorylation site in FGFR1 has previously been mapped to tyrosine 766 in the carboxy-terminal region of the receptor, and this has been shown to be a binding site for the SH2 domain of PLCγ (51, 52, 58). We have mapped six new autophosphorylation sites in FGFR1 and have investigated their importance for the regulation of FGFR1 kinase activity and FGFR1-mediated mitogenesis or neuronal differentiation.

Two of the newly identified autophosphorylation sites, tyrosines 653 and 654, are located in a region homologous to Y-416 of $pp60^{c\text{-}src}$, the phosphorylation of which stimulates Src kinase activity (38, 59). A group of tyrosine kinase receptors, including FGF receptor, insulin receptor, insulin-like growth factor I receptor, scatter factor/hepatocyte growth factor receptor (Met), and nerve growth factor receptor (Trk), also have two tyrosine residues in homologous positions (25, 26). In the case of insulin receptor, TrkA, TrkB, and Met, autophosphorylation occurs on both tyrosines and is important both for kinase activity and for mediating biological responses (18, 21, 22, 24, 40, 41, 46, 50, 54, 62, 72, 80, 86). The three-dimensional structure of insulin receptor kinase was determined by X-ray crystallography, and it has been proposed that the kinase is autoinhibited by the presence of tyrosine 1162 in the active site of the catalytic domain. This would interfere with the binding of exogenous substrates as well as with the binding of Mg-ATP (30). Tyrosine 653 of FGFR1 is in the position homologous to 1162 of insulin receptor and therefore may play a similar role in regulation of the kinase activity of FGFR1. However, our results indicate that mutation of tyrosine 653 alone does not affect the kinase activity of FGFR1. We demonstrate that the presence of both autophosphorylated tyrosines 653 and 654 in FGFR1 is necessary for full kinase activity in vitro and for its biological responses in vivo. Mutation of Y-654 alone reduces the kinase activity of FGFR1 in vitro and diminishes FGFR1-mediated mitogenesis in L-6 cells and neuronal differentiation

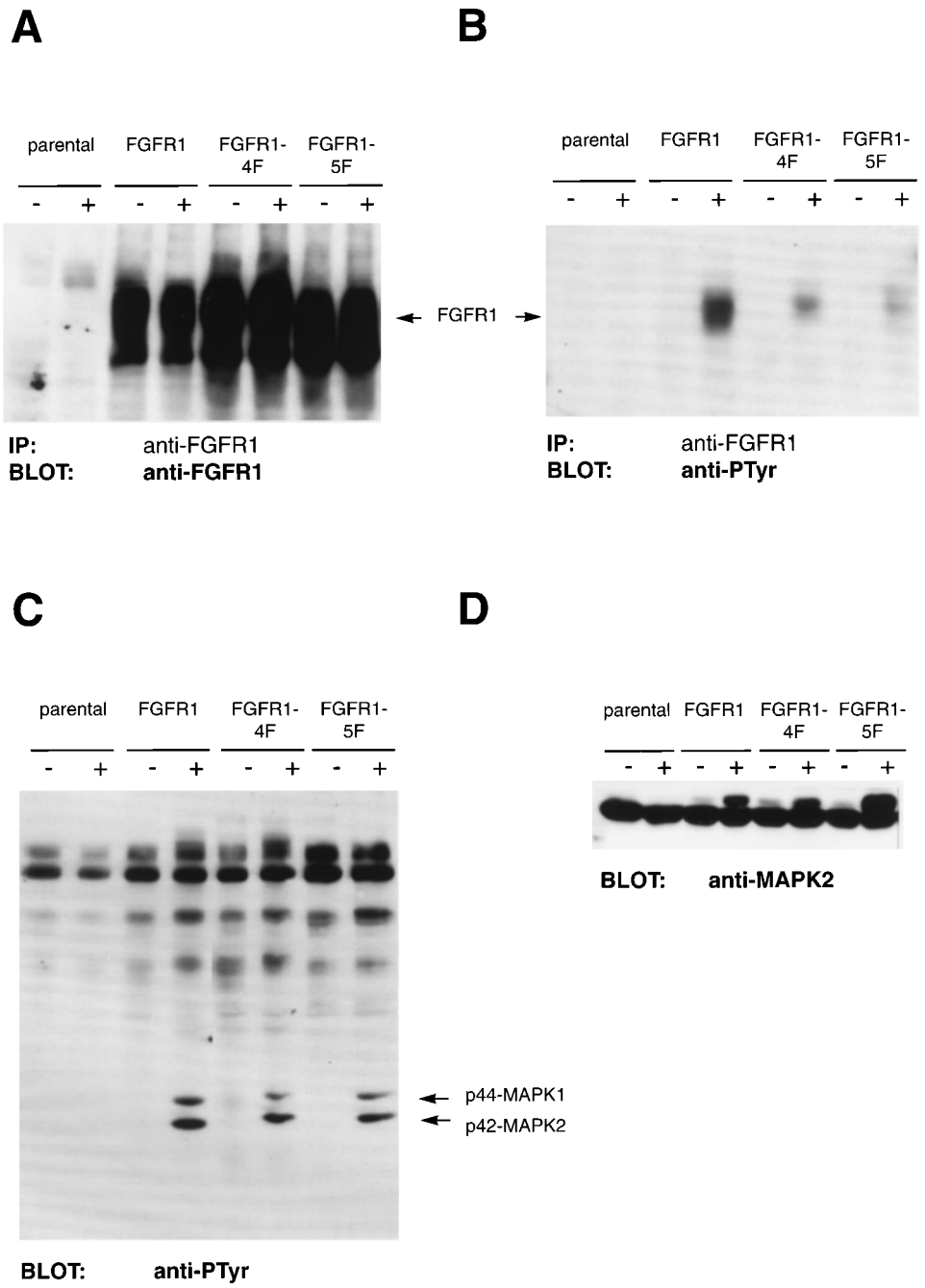

FIG. 5. Mutation of tyrosines 463, 583, 585, 730, and 766 on FGFR1 does not affect MAP kinase activation in response to aFGF. Expression (A) and tyrosine autophosphorylation (B) of wild-type and mutant FGF receptors in L-6 myoblasts. Anti-FGFR1 immunoprecipitates (IP) from aFGF-treated (+) or unstimulated (−) cells expressing wild-type FGFR1 or the mutant FGFR1-4F and FGFR1-5F receptors were analyzed by immunoblotting with either anti-FGFR1 (A) or anti-phosphotyrosine (anti-PTyr) (B) antibodies. (C) Comparison of tyrosine phosphorylation of intracellular proteins induced by aFGF in cells expressing mutant receptors. Lysates from aFGF-treated or untreated cells expressing wild-type FGFR1 and FGFR1-4F and FGFR1-5F mutant receptors were analyzed by SDS-PAGE and immunoblotting with anti-PTyr antibodies. The positions of the 42- and 44-kDa MAP kinases are indicated by arrows. (D) Activation of MAP kinase in response to aFGF in cells expressing wild-type or mutant FGFR1. Cells were stimulated with aFGF (100 ng/ml) for 5 min and lysed, and lysates were analyzed by SDS-PAGE and immunoblotting with anti-MAP kinase 2 (anti-MAPK2) antibodies.

in PC12 cells. However, mutation of tyrosine 653 to phenylalanine is dispensable for both kinase activity and biological responses of FGFR1. Although autophosphorylation on tyrosine 653 of FGFR1 was reported previously (28, 65), phosphorylation of tyrosine 654 has not been described. Furthermore, the role of these tyrosines in activation of the kinase activity was not tested. Tyrosines 653 and 654 are conserved in all known members of the FGF receptor family, and we propose that homologous tyrosines in other FGF receptors are also important for activation of protein tyrosine kinase activity.

In addition, we have identified tyrosine 463 in the juxtamembrane region, tyrosines 583 and 585 in the kinase insert region,

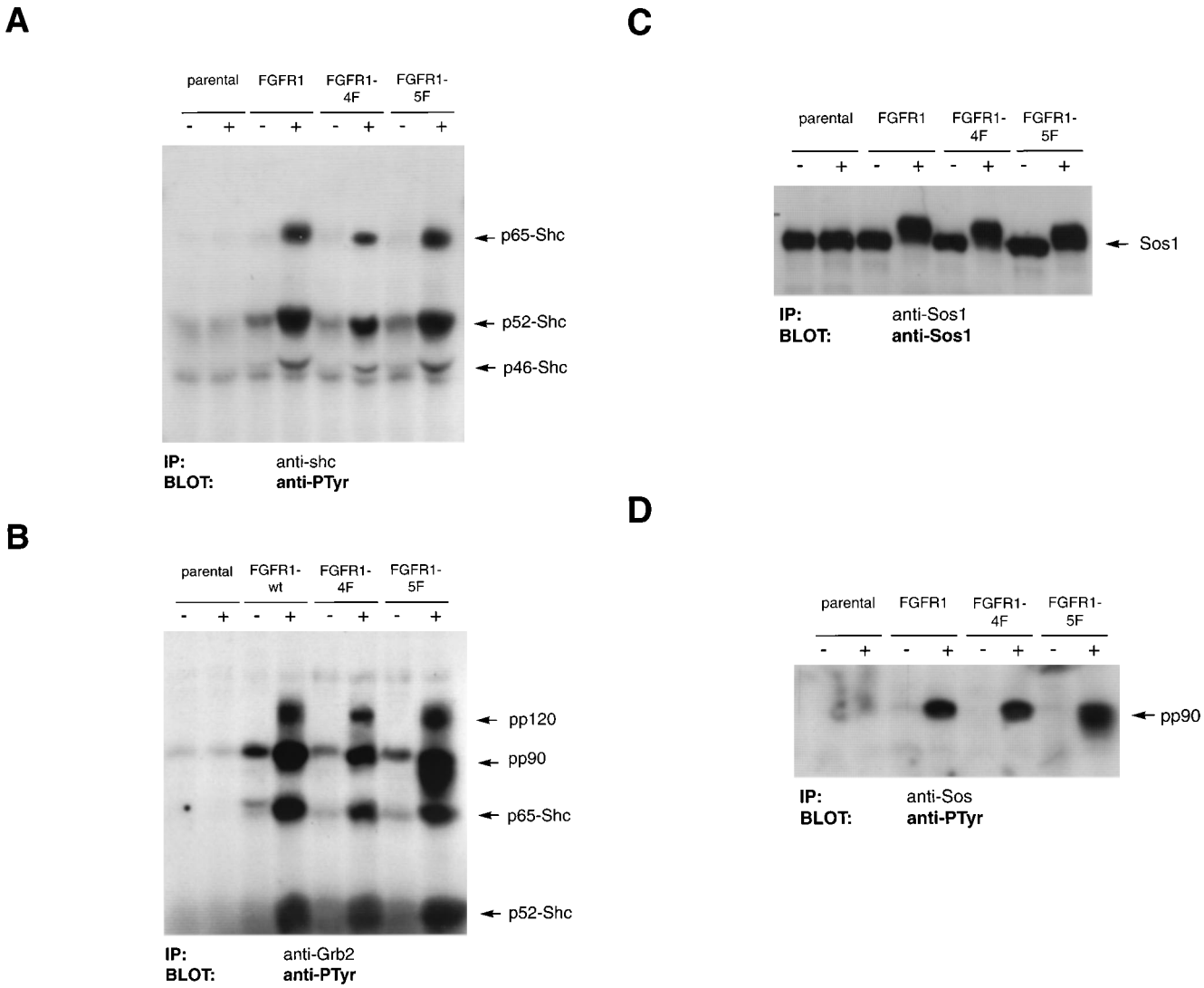

FIG. 6. Shc and pp90 are tyrosine phosphorylated and associate with Grb2 in response to aFGF stimulation of FGFR1-5F. Comparison of tyrosine phosphorylation of Shc (A) and Grb2-associated pp90 (B) in response to aFGF in cells expressing wild-type and mutant FGF receptors. Stable cell lines expressing wild-type FGFR1 and the FGFR1-4F and FGFR1-5F mutant receptors were treated with aFGF (+) or left untreated (−). Lysates were then immunoprecipitated with anti-Shc (A), anti-Grb-2 (B), or anti-Sos (C and D) antibodies and analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (anti-PTyr) (A, B, and D) or anti-Sos antibodies (C). The small differences seen in tyrosine phosphorylation of Shc or pp90 are not significant. Comparable tyrosine phosphorylation of Shc or pp90 was obtained with different cell lines expressing each mutant receptor. IP, immunoprecipitate.

and tyrosine 730 in the second half of the conserved kinase domain as four novel autophosphorylation sites of FGFR1. Tyrosines 463, 583, and 585 are conserved among several FGF receptors of human, mouse, chicken, *Xenopus*, *Drosophila*, and *Caenorhabditis elegans* origin (15, 31, 37, 66). Autophosphorylation sites in the juxtamembrane region of PDGF receptor were shown to be involved in the activation of Src family kinases (53). Furthermore, autophosphorylation sites in the juxtamembrane regions of insulin receptor and Trk are important for tyrosine phosphorylation of insulin receptor substrate 1 or Shc, respectively (55, 72, 80). We as well as others have not been able to demonstrate any direct interaction for activated FGFR1 with Src or with Shc in vivo (reference 43 and our unpublished observation), although there is one report describing association of Src with FGFR1 in vitro (85). Tyrosine phosphorylation sites in the kinase insert region have been mapped for several class III receptors, such as the PDGF and colony-stimulating factor 1 receptors and have been shown to be responsible for the activation of PI 3-kinase (8, 57). The autophosphorylation sites in the kinase insert region of the FGF receptor do not have any homology to known SH2 or PTB domain binding consensus sequence motifs. Tyrosine 730 is located in the carboxy-terminal part of the second half of the kinase domain and is conserved in the FGF receptors of all species so far cloned (31, 34). This tyrosine is also found in other receptors with tyrosine kinase activity, such as epidermal growth factor, PDGF, colony-stimulating factor 1, and stem cell factor receptors but is not present in the insulin receptor (25). However, autophosphorylation of this tyrosine in these receptors has not been reported. This tyrosine has a YMXM motif which represents a consensus binding site for the SH2 domain of regulatory subunit of PI 3-kinase p85 (8). However, we were not able to coimmunoprecipitate FGFR1 with p85 or to detect any associated PI 3-kinase activity in anti-FGFR1

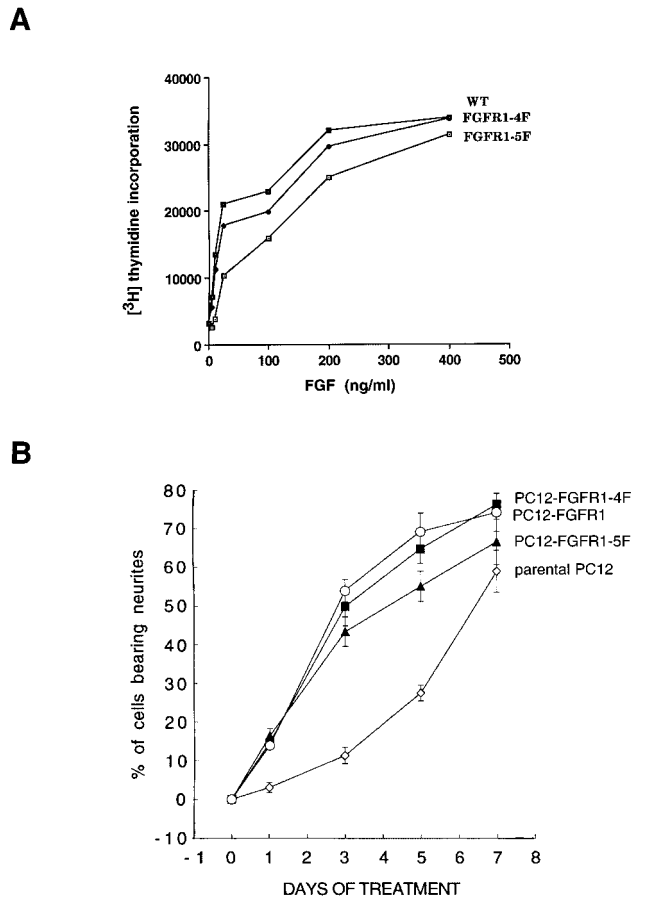

FIG. 7. Autophosphorylation on tyrosines 463, 583, 585, 730, and 766 is dispensable for aFGF-induced mitogenesis and neuronal differentiation. (A) Thymidine incorporation in response to aFGF in cells expressing wild-type (WT) and mutant FGF receptors. Stable cell lines expressing wild-type FGFR1 or the FGFR1-4F and FGFR1-5F mutant receptors were starved for 48 h in the presence of 0.1% FBS and then treated with different concentrations of aFGF or, as a control, with 10% FBS for 24 h. [^{3}H]thymidine was added, and after an additional 16 h the amount of incorporated [^{3}H]thymidine was determined. (B) aFGF induces neuronal differentiation of PC12 cells expressing wild-type FGFR1 or FGFR1-4F and FGFR1-5F mutant receptors. Parental PC12 cells or PC12 cell lines expressing wild-type (PC12-FGFR1) or mutant (PC12-FGFR1-4F and PC12-FGFR1-5F) receptors were stimulated with aFGF (100 ng/ml) and scored for neurite outgrowth as a function of time. The weaker response of parental PC12 cells is due to the low level of wild-type FGF receptors expressed in these cells. The datum points represent the means of three independent experiments (bars, standard errors).

immunoprecipitates (reference 79 and our unpublished observation).

Using mutational analysis, we investigated the contribution of these autophosphorylation sites to signaling via FGFR1. Several experiments have demonstrated that activation of the Ras/MAP kinase signaling pathway is crucial for FGFR1-mediated biological responses (42, 47, 61, 82, 83). Our results show that removal of all the identified autophosphorylation sites in FGFR1, except those involved in the activation of receptor tyrosine kinase activity (Y-653 and Y-654), does not perturb the ability of FGFR1 to recruit the Grb2/Sos complex via tyrosine phosphorylation of Shc and/or pp90 or to activate the MAP kinase cascade.

This observation contrasts with studies of other growth factor receptors, such as PDGF receptor, colony-stimulating factor 1 receptor, Trk, and Met, for which mutation of certain autophosphorylation sites to phenylalanine impaired the ability to exert biological effects such as mitogenesis, transformation, or PC12 cell neuronal differentiation (19, 60, 72, 76, 77). Thus, certain autophosphorylated tyrosines in these growth factor receptors are responsible for recruitment of signaling molecules essential for the activation of the Ras/MAP kinase signaling pathway. Elimination of these sites abolishes the induction of mitogenesis or differentiation by the respective ligands. An alternative mechanism appears to be employed by other receptor tyrosine kinases, such as the insulin receptor. The insulin receptor undergoes autophosphorylation on tyrosines but does not directly bind SH2 domain-containing proteins in vivo. Instead, the insulin receptor phosphorylates other cellular proteins such as Shc and insulin receptor substrate 1, which then serve as docking proteins for SH2 domain-containing proteins (1a, 67, 68).

Our previous reports and the results presented in this study suggest that autophosphorylation of FGFR1 has multiple roles in signaling. Autophosphorylated tyrosines 653 and 654 regulate the kinase activity of FGFR1 and therefore are necessary for all responses mediated by the kinase domain of FGFR1 (Fig. 8). Tyrosine 766 is a binding site for the SH2 domain of PLCγ and couples FGFR1 to PI metabolism and Ca^{2+} mobilization but is not required for the activation of the Ras/MAP kinase signaling pathway or Ras-dependent biological responses (Fig. 8). The remaining four autophosphorylated tyrosines are not necessary for FGF-induced activation of the Ras/MAP kinase signaling cascade, mitogenesis in L-6 cells, or

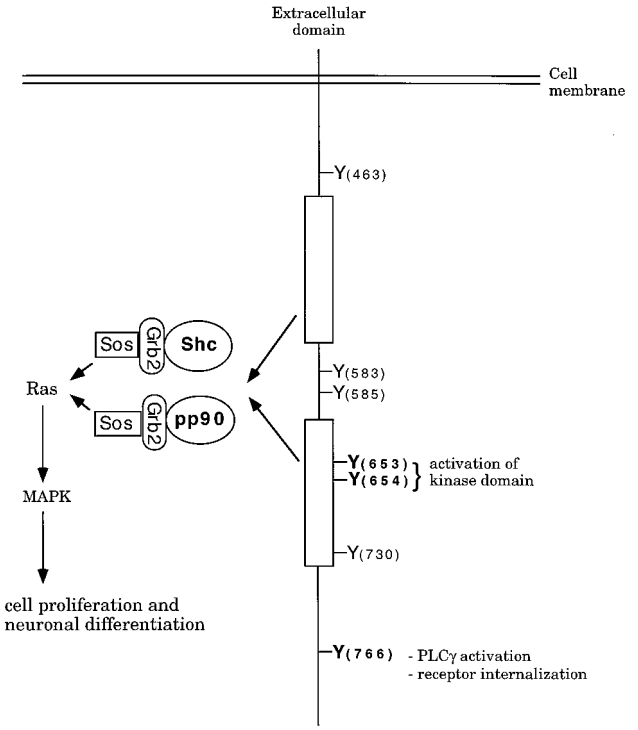

FIG. 8. Schematic representation of all known tyrosine autophosphorylation sites on FGFR1. The seven tyrosine autophosphorylation sites identified on FGFR1 (*flg*) so far are shown. Autophosphorylation on tyrosines 653 and 654 activates the FGF receptor kinase domain. Autophosphorylation on tyrosine 766 couples FGF receptor to PLCγ activation, which then leads to influx of Ca^{2+} and activation of protein kinase C. While this pathway is not required for aFGF-induced mitogenesis, it is involved in the internalization of FGFR1. The function of the remaining autophosphorylation sites, 463, 583, 585, and 730, remains unclear. MAPK, MAP kinase.

neuronal differentiation in PC12 cells (Fig. 8). It was recently demonstrated that a *C. elegans* homolog of FGFR is required for normal migrations of the sex myoblasts in *C. elegans* hermaphrodites (15). It is possible that these autophosphorylation sites are involved in plasminogen activation and chemotaxis in response to aFGF stimulation. We were not able to demonstrate association of any known SH2 domain-containing proteins, except for PLCγ, with the activated FGFR1 in several cell types. This could be due to a weak or transient interaction between FGFR1 and its targets, or alternatively this may suggest that the remaining autophosphorylated tyrosines do not serve as docking sites for the known SH2 domain-containing proteins. The mechanism of FGFR1-induced activation of the Ras/MAP kinase signaling cascade is not entirely clear at this moment. We have noticed tyrosine phosphorylation of Shc and an as yet unidentified 90-kDa protein (pp90) upon stimulation of wild-type or mutant FGFR1 (FGFR1-5F). Tyrosine-phosphorylated Shc binds the Grb2/Sos complex and could link FGFR1 to the Ras signaling pathway (71). In addition, we have demonstrated that tyrosine-phosphorylated pp90 associates with the Grb2/Sos complex and may alternatively link FGFR1 to the Ras signaling pathway. Therefore, FGFR1 may signal in a manner somewhat similar to that of the insulin receptor, by employing docking molecules such as Shc or p90 to couple to the Ras and other signaling pathways (Fig. 8).

ACKNOWLEDGMENTS

M.M. and I.D. contributed equally to this work.

We thank S. Hubbard and M. Lemmon for critical reading of this manuscript and helpful suggestions. The secretarial help of Janice Small is acknowledged.

This work was supported by a grant from Sugen Inc. (to J.S.).

REFERENCES

1. **Allen, G.** 1989. Sequencing of proteins and peptides, 2nd ed. Elsevier, New York.
1a. **Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White.** 1992. Phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. **11:**3469–3479.
2. **Batzer, A. G., D. Rotin, J. M. Urena, E. Y. Skolnik, and J. Schlessinger.** 1994. Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol. Cell. Biol. **14:**5192–5201.
3. **Bellot, F., G. Crumley, J. Kaplow, J. Schlessinger, M. Jaye, and C. A. Dionne.** 1991. Ligand-induced transphosphorylation between different FGF receptors. EMBO J. **10:**2849–2854.
4. **Blaikie, P., D. Immanuel, J. Wu, N. Li, V. Yajnik, and B. Margolis.** 1994. A region distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. J. Biol. Chem. **269:**32031–32034.
5. **Bork, P., and B. Margolis.** 1995. A new phosphotyrosine interaction domain. Cell **80:**693–694.
6. **Burgess, W. H., C. A. Dionne, J. Kaplow, R. Mudd, R. Friesel, A. Zilberstein, J. Schlessinger, and M. Jaye.** 1990. Characterization and cDNA cloning of phospholipase C-γ, a major substrate for heparin-binding growth factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. Mol. Cell. Biol. **10:**4770–4777.
7. **Burgess, W., and T. Maciag.** 1989. Heparin binding (fibroblast) growth factor family of proteins. Annu. Rev. Biochem. **58:**575–606.
8. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64:** 281–302.
9. **Chellaiah, A. T., D. G. McEwen, S. Werner, J. Xu, and D. M. Ornitz.** 1994. Fibroblast growth factor receptor (FGFR3). Alternative splicing in immunoglobulin-like domain III creates a receptor highly specific for acidic FGF/FGF-1. J. Biol. Chem. **269:**11620–11627.
10. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
11. **Clyman, R. I., K. G. Peters, Y. Q. Chen, J. Escobedo, L. T. Williams, H. E. Ives, and E. Wilson.** 1994. Phospholipase C gamma activation, phosphatidylinositol hydrolysis, and calcium mobilization are not required for FGF receptor-mediated chemotaxis. Cell Adhesion Commun. **1:**333–342.
12. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99:**387–402.
13. **Coughlin, S. R., P. J. Barr, L. S. Cousens, L. J. Fretto, and L. T. Williams.** 1988. Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. J. Biol. Chem. **263:**988–993.
14. **Crumley, G., F. Bellot, J. M. Kaplow, J. Schlessinger, M. Jaye, and C. A. Dionne.** 1991. High-affinity binding and activation of a truncated FGF receptor by both aFGF and bFGF. Oncogene **6:**2255–2262.
15. **DeVore, D. L., H. R. Horvitz, and M. J. Stern.** 1995. An FGF receptor signaling pathway is required for the normal cell migrations of the sex myoblasts in C. elegans hermaphrodites. Cell **83:**611–620.
16. **Dikic, I., A. G. Batzer, P. Blaikie, A. Obermeier, A. Ullrich, J. Schlessinger, and B. Margolis.** 1995. Shc binding to NGF receptor is mediated by the PI domain. J. Biol. Chem. **270:**15125–15129.
17. **Dikic, I., J. Schlessinger, and I. Lax.** 1994. PC12 cells overexpressing the insulin receptor undergo insulin dependent neuronal differentiation. Curr. Biol. **4:**702–708.
18. **Ellis, L., E. Clauser, D. O. Morgan, E. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45:**721–732.
19. **Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormick, and L. T. Williams.** 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell **69:**413–423.
20. **Fantl, W. J., D. E. Johnson, and L. T. Williams.** 1993. Signaling by receptor tyrosine kinases. Annu. Rev. Biochem. **62:**453–481.
21. **Ferracini, R., P. Longati, L. Naldini, E. Vigna, and P. M. Comoglio.** 1991. Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. J. Biol. Chem. **266:**19558–19564.
22. **Flores-Riveros, J. R., E. Sibley, T. Kastelic, and M. D. Lane.** 1989. Substrate phosphorylation catalyzed by the insulin receptor tyrosine kinase. Kinetic correlation to autophosphorylation of specific sites in the beta subunit. J. Biol. Chem. **264:**21557–21572.
23. **Friesel, R., W. H. Burgess, and T. Maciag.** 1989. Heparin-binding growth factor 1 stimulates tyrosine phosphorylation in NIH 3T3 cells. Mol. Cell. Biol. **9:**1857–1865.
24. **Guiton, M., F. J. Gunn-Moore, T. N. Stitt, G. D. Yancopoulos, and J. M. Tavare.** 1994. Identification of in vivo brain-derived neurotrophic factor-stimulated autophosphorylation sites on the TrkB receptor tyrosine kinase by site-directed mutagenesis. J. Biol. Chem. **269:**30370–30377.
25. **Hanks, S. K.** 1991. Eukaryotic protein kinases. Curr. Opin. Struct. Biol. **1:** 369–383.
26. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:**42–52.
27. **Hou, J., M. Kan, K. McKeehan, G. McBride, P. Adams, and W. L. McKeehan.** 1991. Fibroblast growth factor receptors from liver vary in three structural domains. Science **251:**665–668.
28. **Hou, J., K. McKeehan, M. Kan, S. A. Carr, M. J. Huddeston, J. W. Crabb, and W. L. McKeehan.** 1993. Identification of tyrosines 154 and 307 in the extracellular domain and 653 and 766 in the intracellular domain as phosphorylation sites in the heparin-binding fibroblast growth factor receptor tyrosine kinase (*flg*). Protein Sci. **2:**86–92.
29. **Huang, J., M. Mohammadi, G. A. Rodrigues, and J. Schlessinger.** 1995. Reduced activation of RAF-1 and MAP kinase by a fibroblast growth factor receptor mutant deficient in stimulation of phosphatidylinositol hydrolysis. J. Biol. Chem. **270:**5065–5072.
30. **Hubbard, S. R., L. Wei, L. Ellis, and W. A. Hendrickson.** 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature (London) **372:**746–754.
31. **Jaye, M., J. Schlessinger, and C. A. Dionne.** 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. Biochim. Biophys. Acta **1135:**185–199.
32. **Johnson, D. E., P. L. Lee, J. Lu, and L. T. Williams.** 1990. Diverse forms of a receptor for acidic and basic fibroblast growth factors. Mol. Cell. Biol. **10:** 4728–4736.
33. **Johnson, D. E., J. Lu, H. Chen, S. Werner, and L. T. Williams.** 1991. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. Mol. Cell. Biol. **11:**4627–4634.
34. **Johnson, D. E., and L. T. Williams.** 1993. Structural and functional diversity in the FGF receptor multigene family. Adv. Cancer Res. **60:**1–41.
35. **Kavanaugh, W. M., C. W. Turck, and L. T. Williams.** 1995. PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. Science **268:**1177–1179.
36. **Kavanaugh, W. M., and L. T. Williams.** 1994. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science **266:**1862–1865.
37. **Klambt, C., L. Glazer, and B. C. Shilo.** 1992. Breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. Genes Dev. **6:**1668–1678.
38. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49:**65–73.
39. **Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2

and SH3 domains: elements that control interaction of cytoplasmic signaling proteins. Science **252:**668–674.
40. **Kohanski, R. A.** 1993. Insulin receptor autophosphorylation. II. Determination of autophosphorylation sites by chemical sequence analysis and identification of the juxtamembrane sites. Biochemistry **32:**5773–5780.
41. **Komada, M., and N. Kitamura.** 1994. Regulatory role of major tyrosine autophosphorylation site of kinase domain of c-Met receptor (scatter factor/hepatocyte growth factor receptor). J. Biol. Chem. **269:**16131–16136.
42. **Kremer, N. E., G. D'Arcangelo, S. M. Thomas, M. De Marco, J. S. Brugge, and S. Halegoua.** 1991. Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of Src and Ras actions. J. Cell Biol. **115:**809–819.
43. **Landgren, E., P. Blume-Jensen, S. A. Courtneidge, and L. Claesson-Welsh.** 1995. Fibroblast growth factor receptor-1 regulation of Src family kinases. Oncogene **10:**2027–2035.
44. **Li, N., A. Batzer, R. Daly, V. Yajnik, E. Y. Skolnik, P. Chardin, D. Bar-Sagi, B. Margolis, and J. Schlessinger.** 1993. Guanine nucleotide releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinase to Ras signaling. Nature (London) **363:**85–88.
45. **Li, N., J. Schlessinger, and B. Margolis.** 1994. Autophosphorylation mutants of the EGF-receptor signal through auxiliary mechanisms involving SH2 domain proteins. Oncogene **9:**3457–3465.
46. **Longati, P., A. Bardelli, C. Ponzetto, L. Naldini, and P. M. Comoglio.** 1994. Tyrosines 1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene **9:**49–57.
47. **MacNicol, A. M., A. J. Muslin, and L. T. Williams.** 1993. Raf-1 kinase is essential for early Xenopus development and mediates the induction of mesoderm by FGF. Cell **73:**571–583.
48. **Margolis, B.** 1992. Proteins with SH2 domains: transducers in the tyrosine kinase signaling pathway. Cell Growth Differ. **3:**73–80.
49. **Margolis, B., A. Zilberstein, C. Franks, S. Felder, S. Kremer, A. Ullrich, S. G. Rhee, K. Skorecki, and J. Schlessinger.** 1990. Effect of phospholipase C-gamma overexpression on PDGF-induced second messengers and mitogenesis. Science **248:**607–610.
50. **Middlemas, D. S. J. Meisenhelder, and T. Hunter.** 1994. Identification of TrkB autophosphorylation sites and evidence that phospholipase C-gamma 1 is a substrate of the TrkB receptor. J. Biol. Chem. **269:**5458–5466.
51. **Mohammadi, M., C. A. Dionne, W. Li, N. Li, T. Spivak, A. M. Honegger, M. Jaye, and J. Schlessinger.** 1992. Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature (London) **358:**681–684.
52. **Mohammadi, M., A. M. Honegger, D. Rotin, R. Fisher, F. Bellot, W. Li, C. A. Dionne, M. Jaye, M. Rubinstein, and J. Schlessinger.** 1991. A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (*flg*) is a binding site for the SH2 domain of phospholipase C-γ1. Mol. Cell. Biol. **11:**5068–5078.
53. **Mori, S., L. Ronnstrand, K. Yokote, A. Engstrom, S. A. Courtneidge, L. Claesson-Welsh, and C. H. Heldin.** 1993. Identification of two juxtamembrane autophosphorylation sites in the PDGF beta-receptor; involvement in the interaction with Src family tyrosine kinases. EMBO J. **12:**2257–2264.
54. **Naldini, L., E. Vigna, R. Ferracini, P. Longati, L. Gandino, M. Prat, and P. M. Comoglio.** 1991. The tyrosine kinase encoded by the MET proto-oncogene is activated by autophosphorylation. Mol. Cell. Biol. **11:**1793–1803.
55. **Obermeier, A., R. A. Bradshaw, K. Seedorf, A. Choidas, J. Schlessinger, and A. Ullrich.** 1994. Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma. EMBO J. **13:**1585–1590.
56. **Pawson, T.** 1995. Protein modules and signaling networks. Nature (London) **373:**573–580.
57. **Pawson, T., and J. Schlessinger.** 1993. SH2 and SH3 domains. Curr. Biol. **3:**434–442.
58. **Peters, K. G., J. Marie, E. Wilson, H. E. Ives, J. Escobedo, M. Del Rosario, D. Mirda, and L. T. Williams.** 1992. Point mutation of an FGF receptor abolishes phospatidylinositol turnover and Ca2+ flux but not mitogenesis. Nature (London) **358:**678–781.
59. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60c-src. Cell **49:**75–82.
60. **Ponzetto, C., A. Bardelli, Z. Zhen, F. Maina, P. dalla Zonca, S. Giordano, A. Graziani, G. Panayotou, and P. M. Comoglio.** 1994. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell **77:**261–271.
61. **Reichman-Fried, M., B. Dickson, E. Hafen, and B. Z. Shilo.** 1994. Elucidation of the role of breathless, a Drosophila FGF receptor homolog, in tracheal cell migration. Genes Dev. **8:**428–439.
62. **Rodrigues, G. A., and M. Park.** 1994. Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase. Oncogene **9:**2019–2027.
63. **Rosen, O. M., R. Herrera, Y. Olowe, L. M. Petruzzelli, and M. H. Cobb.** 1983. Phosphorylation activates the insulin receptor tyrosine protein kinase. Proc. Natl. Acad. Sci. USA **80:**3237–3240.
64. **Schlessinger, J., and A. Ullrich.** 1992. Growth factor signaling by receptor tyrosine kinases. Neuron **9:**383–391.
65. **Shi, E., M. Kan, J. Xu, F. Wang, J. Hou, and W. L. McKeehan.** 1993. Control of fibroblast growth factor receptor kinase signal transduction by heterodimerization of combinatorial splice variants. Mol. Cell. Biol. **13:**3907–3918.
66. **Shishido, E., S. Higashijima, Y. Emori, and K. Saigo.** 1993. Two FGF-receptor homologues of Drosophila: one is expressed in mesodermal primordium in early embryos. Development **117:**751–761.
67. **Skolnik, E. Y., A. Batzer, N. Li, C. H. Lee, E. Lowenstein, M. Mohammadi, B. Margolis, and J. Schlessinger.** 1993. The function of Grb2 in linking the insulin receptor to Ras signaling pathway. Science **260:**1953–1955.
68. **Skolnik, E. Y., C. H. Lee, A. G. Batzer, L. M. Vicentini, M. Zhou, R. Daly, M. J. Myers, J. M. Backer, A. Ullrich, M. F. White, and J. Schlessinger.** 1993. The SH2/SH3 domain containing protein Grb2 interacts with tyrosine phosphorylated IRS1 and Shc: implications for insulin control of Ras signaling. EMBO J. **12:**1929–1936.
69. **Sorokin, A., M. Mohammadi, J. Huang, and J. Schlessinger.** 1994. Internalization of fibroblast growth factor is inhibited by a point mutation at tyrosine 766. J. Biol. Chem. **269:**17056–17061.
70. **Spivak-Kroizman, T., M. A. Lemmon, I. Dikic, J. E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlessinger, and I. Lax.** 1994. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. Cell **79:**1015–1024.
71. **Spivak-Kroizman, T., M. Mohammadi, P. Hu, M. Jaye, J. Schlessinger, and I. Lax.** 1994. Point mutation in the fibroblast growth factor receptor eliminates phosphatidylinositol hydrolysis without affecting neuronal differentiation of PC12 cells. J. Biol. Chem. **269:**14419–14423.
72. **Stephens, R. M., D. M. Loeb, T. D. Copeland, T. Pawson, L. A. Green, and D. R. Kaplan.** 1994. Trk receptors use redundant signal transduction pathways involving Shc and PLC-gamma 1 to mediate NGF responses. Neuron **12:**691–705.
73. **Ueno, H., M. Gunn, K. Dell, A. Tseng, and L. T. Williams.** 1992. A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. J. Biol. Chem. **267:**1470–1476.
74. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212.
75. **Vainikka, S., V. Joukov, S. Wennstrom, M. Bergman, P. G. Pelicci, and K. Alitalo.** 1994. Signal transduction by fibroblast growth factor receptor-4 (FGFR-4). Comparison with FGFR-1. J. Biol. Chem. **269:**18320–18326.
76. **Valius, M., and A. Kazlauskas.** 1993. Phospholipase C-gamma 1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. Cell **73:**321–334.
77. **Van der Geer, P., and T. Hunter.** 1993. Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. EMBO J. **12:**5161–5172.
78. **Wang, J. K., G. Gao, and M. Goldfarb.** 1994. Fibroblast growth factor receptors have different signaling and mitogenic potentials. Mol. Cell. Biol. **14:**181–188.
79. **Wennstroem, S., E. Landgren, P. Blume-Jensen, and L. Claesson-Welsh.** 1992. The platelet-derived growth factor β-receptor kinase insert confers specific signaling properties to a chimeric fibroblast growth factor receptor. J. Biol. Chem. **267:**13749–13756.
80. **White, M. F., J. N. Livingston, J. M. Backer, V. Lauris, T. J. Dull, A. Ullrich, and C. R. Kahn.** 1988. Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. Cell **54:**641–649.
81. **White, M. F., S. E. Shoelson, H. Keutmann, and C. R. Kahn.** 1988. A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. J. Biol. Chem. **263:**2969–2980.
82. **Whitman, M., and D. A. Melton.** 1992. Involvement of p21ras in Xenopus mesoderm induction. Nature (London) **357:**252–254.
83. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68:**1041–1050.
84. **Yu, K. T., and M. P. Czech.** 1984. Tyrosine phosphorylation of the insulin receptor beta subunit activates the receptor-associated tyrosine kinase activity. J. Biol. Chem. **259:**5277–5286.
85. **Zhan, X., C. Plourde, X. Hu, R. Friesel, and T. Maciag.** 1994. Association of fibroblast growth factor receptor-1 with c-Src correlates with association between c-Src and cortactin. J. Biol. Chem. **269:**20221–20224.
86. **Zhang, B., J. M. Tavare, L. Ellis, and R. A. Roth.** 1991. The regulatory role of known tyrosine autophosphorylation sites of the insulin receptor kinase domain. An assessment by replacement with neutral and negatively charged amino acids. J. Biol. Chem. **266:**990–996.