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MOLECULAR AND CELLULAR BIOLOGY, May 2008, p. 3372–3385 Vol. 28, No. 10
0270-7306/08/$08.00+0 doi:10.1128/MCB.01837-07
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Fibroblast Growth Factor Receptor 2 Phosphorylation on Serine 779 Couples to 14-3-3 and Regulates Cell Survival and Proliferation[▽]

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Received 10 October 2007/Returned for modification 26 November 2007/Accepted 15 February 2008

The fibroblast growth factors (FGFs) exert their diverse (or pleiotropic) biological responses through the binding and activation of specific cell surface receptors (FGFRs). While FGFRs are known to initiate intracellular signaling through receptor tyrosine phosphorylation, the precise mechanisms by which the FGFRs regulate pleiotropic biological responses remain unclear. We now identify a new mechanism by which FGFR2 is able to regulate intracellular signaling and cellular responses. We show that FGFR2 is phosphorylated on serine 779 (S779) in response to FGF2. S779, which lies adjacent to the phospholipase Cγ binding site at Y766, provides a docking site for the 14-3-3 phosphoserine-binding proteins and is essential for the full activation of the phosphatidylinositol 3-kinase and Ras/mitogen-activated protein kinase pathways. Furthermore, S779 signaling is essential for promoting cell survival and proliferation in both Ba/F3 cells and BALB/c 3T3 fibroblasts. This new mode of FGFR2 phosphoserine signaling via the 14-3-3 proteins may provide an increased repertoire of signaling outputs to allow the regulation of pleiotropic biological responses. In this regard, we have identified conserved putative phosphotyrosine/phosphoserine motifs in the cytoplasmic domains of diverse cell surface receptors, suggesting that they may perform important functional roles beyond the FGFRs.

The fibroblast growth factors (FGFs) constitute a large family of proteins (22 members) that mediate diverse (or pleiotropic) cellular responses such as cell survival, proliferation, differentiation, and migration (7, 9). The pleiotropic activities mediated by FGFs are essential for the regulation of such dynamic processes as embryogenesis, wound healing, tissue repair, and angiogenesis (9). The FGFs exert their biological activities through the binding and activation of four structurally related FGF receptors (FGFR): FGFR1 (Flg), FGFR2 (Bek), FGFR3, and FGFR4, each of which is subject to alternative mRNA splicing to generate receptors with distinct ligand-binding specificities (37). The prototypic members of the FGF family of growth factors, FGF1 (acidic FGF) and FGF2 (basic FGF), are able to bind FGFR1 and FGFR2, leading to receptor dimerization/oligomerization, activation of intrinsic receptor tyrosine kinase activity, and the phosphorylation of specific tyrosine residues in the cytoplasmic tail of the receptor (9). As widely observed for other receptor tyrosine kinases (RTKs), phosphotyrosine residues in the cytoplasmic domains of FGFR1 and FGFR2 provide docking sites for Src-homology 2 (SH2) domain proteins and allow the activated receptor to physically couple to downstream signaling pathways (45, 54, 58).

In the case of FGFR1, seven tyrosine residues in the cytoplasmic domain have been identified as phosphorylation sites: Y463 in the juxtamembrane domain (13, 29, 34), Y583 and Y585 in the kinase insert domain (13, 21, 34), Y653 and Y654 in the activation loop of the kinase domain (13, 21, 34), and Y730 (34) and Y766 (29, 35) in the C-terminal lobe. Of these seven tyrosine residues, Y653 and Y654 are essential for allosteric regulation of kinase activity (34), while the remaining five are either known or putative docking sites for SH2 domain proteins. Given that all seven phosphorylation sites identified in FGFR1 are conserved in FGFR2, a similar tyrosine phosphorylation profile would be expected for FGFR2.

Despite the identification of multiple FGFR tyrosine phosphorylation sites, it has proved difficult to functionally link individual FGFR1 or FGFR2 tyrosine residues to specific cellular responses. For example, Y766 has been shown to act as a binding site for phospholipase Cγ (PLC-γ) (36), and mutation of this site results in a receptor that is defective in PLC-γ activation and phosphatidylinositol (PI) (4, 5) biphosphate hydrolysis (35, 49). In addition, Y766 has also been shown to bind the adaptor proteins Shb (6) and Grb14 (51). However, the precise functional role of Y766 in mediating specific cellular responses remains unclear. Despite being essential for PI hydrolysis and calcium flux, Y766 of FGFR1 was not required for the proliferation of L6 myoblasts, Ba/F3 cells, or porcine aortic endothelial cells (5, 22, 35, 49); the neuronal differentiation of PC12 cells (60); or mesoderm induction in *Xenopus* (38). Although none of these studies identified an essential role for Y766 in mediating specific biological responses, a more recent study suggested that Y766 might play a negative role in FGFR1 signaling. A genetic knock-in of a Y766F mutant of FGFR1 in mice appeared to result in a gain-of-function receptor that led to a partially penetrant phenotype in vertebrate column formation (44). Apart from Y766, a role for the membrane-proximal Y463 in the recruitment of the Crk adaptor protein and the proliferation of porcine aortic endothelial cells has also

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▽ Published ahead of print on 10 March 2008.

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been reported (25). However, other studies utilizing approaches that included domain swapping of the cytoplasmic regions of the FGFRs as well as specific receptor tyrosine mutants suggested that that neither Y463 nor Y766 was necessary for PC12 differentiation (12).

While the FGFRs are able to directly couple to SH2 domain proteins via specific phosphotyrosine docking sites in their cytoplasmic tails, the FGFR substrate 2 (FRS2) proteins also associate with the FGFRs and act as scaffolds for the recruitment of signaling proteins (23). The FRS2 proteins associate with the membrane-proximal region of the FGFRs in a phosphotyrosine-independent manner and then recruit specific SH2 domain proteins such as Grb2, Gab1, and Shp2 (20, 41). Thus, the ability of the FGFRs to recruit signaling proteins through phosphotyrosine residues in their cytoplasmic tails as well as via the FRS2 proteins may impart specific signaling capabilities to regulate a unique spectrum of pleiotropic activities (55).

However, in addition to receptor tyrosine phosphorylation and the recruitment of SH2 and phosphotyrosine binding (PTB) domain proteins, it has recently become apparent that growth factor and cytokine receptors also initiate intracellular signaling via receptor serine/threonine phosphorylation and the recruitment of phosphoserine/threonine binding proteins. While serine/threonine phosphorylation has long been regarded as solely an allosteric regulator of protein activity, the recent discovery of a number of phosphoserine/threonine-binding proteins or modules has radically altered this view (14, 30, 71). For example, the 14-3-3 family of proteins are able to bind phosphoserine/threonine residues within a sequence-specific context (1, 30, 71). Because the 14-3-3 proteins are able to form stable dimers in which each subunit is able to bind a separate phosphoserine peptide, it has been suggested that these proteins may function as adaptors or scaffolds for the assembly of signaling complexes (30). For example, our previous studies have shown that S585 phosphorylation of the βc subunit in the granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) receptors results in binding of the 14-3-3 proteins, the recruitment of PI 3-kinase, and the regulation of hemopoietic cell survival (17–19, 62). While the 14-3-3 proteins have been shown to bind phosphoserine residues in the cytoplasmic tails of a number of other growth factor and cytokine receptors such as the IL-9 receptor α (57), insulin-like growth factor 1 receptor (IGF-1R) (4), the epidermal growth factor (EGF) receptor (39), the prolactin receptor (40), and the transforming growth factor β receptor (33), the biological significance of these 14-3-3–receptor interactions is unclear. In addition to 14-3-3, a number of other phosphoserine/threonine binding domains have been identified including the tryptophan-tryptophan domain (28), the MH2 domains of R-Smads, and the Forkhead-associated, BRCA1 C-terminal, and polo-box domains (14, 30, 69–71). Thus, growth factor receptors may utilize two distinct strategies for the phosphorylation-dependent recruitment of adaptor or scaffold proteins to their cytoplasmic tails: one through receptor tyrosine phosphorylation and the recruitment of SH2 or PTB domain proteins and the other through receptor serine/threonine phosphorylation and the recruitment of phosphoserine/threonine binding proteins such as 14-3-3.

To directly examine this possibility, we scanned the cytoplasmic tails of cell surface receptors for possible 14-3-3 binding sites. We noted that FGFR2 contained a conserved putative 14-3-3 binding site at S779, which lies nearby Y766 in the C-terminal lobe. We developed phospho-specific anti-phospho-S779 antibodies and showed that FGF2 stimulation of Ba/F3 cells expressing FGFR2 results in the phosphorylation of S779. Furthermore, we showed that phospho-S779 was able to bind the 14-3-3 proteins in response to FGF2 stimulation. Expression of an S779G mutant unable to bind the 14-3-3 proteins in either Ba/F3 cells or BALB/c fibroblasts impaired intracellular signaling via the PI 3-kinase and Ras/mitogen-activated protein (MAP) kinase pathways in response to FGF2. Significantly, the S779G mutant was unable to promote cell proliferation of either Ba/F3 cells or BALB/c fibroblasts following FGF2 stimulation. Together, these results identify a new phosphoserine-dependent mechanism by which the FGFRs initiate intracellular signaling pathways leading to specific biological outcomes and further suggest that such novel modes of signaling may serve essential roles downstream of diverse growth factor and cytokine receptors.

MATERIALS AND METHODS

FGFR2 constructs. Y766 and S779 of FGFR2 cDNA (denoted here as BEK) were mutagenized in pBluescript (pB-KS). pB-BEK-HindII/RI was generated by subcloning a HindII/EcoRI fragment from pGC37 into pB. In addition, a 119-bp EcoR1/BglII fragment containing the 3′ coding region of FGFR2 was subcloned into the EcoRI/blunted-BglII site in pB to give pB-BEK-RI/BglII. Tyr766 and Ser779 were mutagenized in pB-BEK-RI/BglII using standard QuickChange mutagenesis. Mutagenic primers were 5′-CTCACAACCAATGAAGAATTTC TGGACCTCAGCCAACC-3′ (Tyr766Phe mutation) and 5′-GAACAGTATTC ACCCGGGTACCCTGACACAAGAAG-3′ (Ser779Gly mutation). An XhoI/EcoRI fragment from pB-BEK-HindIII/RI was then subcloned into pB-BEK-RI/BglII-mutagenized plasmids to give pB-BEK-wt (where wt is wild type), pB-BEK-YF (where YF is yellow fluorescent protein), pB-BEK-SG, and pB-BEK-YF/SG containing full-length BEK cDNA. HindIII/XbaI fragments were then subcloned into the RcCMV (where CMV is cytomegalovirus) plasmid to give RcCMV-BEK-wt, RcCMV-BEK-YF, RcCMV-BEK-SG, and RcCMV-BEK-YF/SG. The complete open reading frames of all plasmids were sequenced to confirm specific mutations.

Antibody production. The anti-phospho-S779 phospho-specific antibody was generated using phospho-peptide encompassing Ser779 of FGFR2 (KEQYSPp SYPDTRC, where pS is phosphoserine) (Chemicon). Reverse-phase high-performance liquid chromatography-purified peptides were subjected to mass spectrometry analysis to confirm correct molecular weights. The phospho-S779 peptide was coupled to keyhole limpet hemocyanin and injected into New Zealand White rabbits. Immunoreactivity was examined from test bleeds using non-phospho-Ser779 and phospho-Ser779 peptides in a dot blot format.

Cell lines. Ba/F3 cells stably expressing RcCMV-wt-FGFR2, FGFR2 carrying the mutation Y766F (FGFR2-Y766F), FGFR2-S779G, and FGFR2-Y766F/S779G were generated by electroporation with individual constructs while BALB/c 3T3 stable transfectants were generated by $CaPO_4$. Transfection was followed by selection in G418 for 2 weeks (Invitrogen). For BALB/c 3T3 cells, individual clones were isolated and examined for their ability to proliferate in response to FGF2 using a Cell Titer 96 colorimetric assay (Promega). Ba/F3 cells were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10% WEHI 3B medium. BALB/c 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

FACS analysis of FGFR2 expression. Cells were pelleted, washed in phosphate-buffered saline (PBS), and resuspended in PBS–0.1% FCS with or without 1 μg of FGFR2 H1 antibody and incubated on ice for 60 min. Cells were washed in PBS three times and incubated with anti-rabbit-fluorescein isothiocyanate (FITC) in PBS–0.1% FCS for 60 min, washed, and analyzed by fluorescence-activated cell sorting (FACS).

Viability assays. Cells were seeded in 96-well plates in the presence or absence of 20 ng/ml FGF2 (R&D Systems). Ba/F3 cells were seeded at 5×10^4 cells per well in RPMI medium containing 0.1% FCS while BALB/c 3T3 cells were plated at 1,000 cells/well in DMEM containing either 10% BIT (stem cell Technologies) or 10% FCS. Viability was assayed following a 24-h culture for Ba/F3 cells and after a 3-day

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culture for BALB/c 3T3 cells by staining with MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] reagent and measuring absorbance at the optical density at 490 nm.

Apoptosis assay. Stably transfected Ba/F3 cells were seeded at 6.7×10^4 cells/well (24-well tray) into RPMI medium containing 0.1% FCS in the presence or absence of 0.5 or 5 ng/ml FGF2. Cells were cultured for 24 h and then pulsed with 200 nM TMRE (tetramethylrhodamine ethyl ester; Invitrogen) for 30 min, washed in PBS, and analyzed by flow cytometry on the FL2 channel. Results are representative of at least three experiments. Error bars indicate standard deviations.

Proliferation assay. Stably transfected Ba/F3 cells were seeded at 2.3×10^4 cells/well (24-well plates) into RPMI medium containing 0.1% FCS in the presence or absence FGF2. Cells were cultured for 24 h and then pulsed with 10 μM bromodeoxyuridine (BrdU; Roche) for 3 h, washed, fixed (3 volumes of 50 mM glycine [pH 2] and 7 volumes of absolute ethanol) for 30 min at 4°C, washed in PBS, resuspended in 4 M HCl, and incubated at room temperature for 20 min. Cells were then washed twice, resuspended in block solution (1:20 dilution of blocking agent [Roche] in PBS) and a 1:10 dilution of anti-BrdU-FITC antibody (Roche), and incubated at 37°C in 5% CO_2 for 45 min. Following staining cells were washed and analyzed by flow cytometry. Results are representative of at least three experiments. Error bars indicate standard deviations.

Analysis of sub-G_1 and cell cycle progression. Cells were fixed in 70% ethanol for 1 h at 4°C, washed, resuspended at a 1:1 ratio of PBS and DNA extraction buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.8), and incubated for 5 min; samples were then centrifuged, resuspended in propidium iodide staining solution (3.8 mM sodium citrate, 50 mg/ml propidium iodide in PBS) with 500 μg of RNase A, and incubated for 30 min. Cells were analyzed by FACS.

Immunoprecipitation, pull-down assays, and Western blotting. Ba/F3 cells (2×10^7 cells/immunoprecipitation) stably expressing wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were factor and serum starved in RPMI medium containing 0.1% FCS for 4 h prior to stimulation with 20 ng/ml FGF2 for the various times. For inhibitor treatments cells were preincubated with inhibitor for 30 min prior to stimulation with FGF. LY294002 (Calbiochem) was used at 33 μM. Wortmannin (Santa Cruz) was used at 100 nM. Rapamycin (Sigma) was used at 1 μM. Glycogen synthase kinase 3 (GSK3) VIII (Calbiochem) was used at 1 μM. BAY11-7082 (Sigma) was used at 100 μM. H89 (Calbiochem) was used at 0.5 μM. SU5402 (Calbiochem) was used at 100 μM. Stably transfected BALB/c 3T3 clones were starved overnight in DMEM containing 0.5% FCS, following which cells were stimulated with 20 ng/ml FGF2 for 10 min. Cells were lysed in NP-40 buffer (10 mM Tris [pH 7.4], 137 mM NaCl, 10% glycerol, 1% Nonidet P-40) supplemented with 10 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 10 mM Na orthovanadate, and 4.5 U/ml aprotinin (Sigma). Cleared lysates were subjected to immunoprecipitation for 2 h at 4°C on a rotating wheel with 1 μg of anti-FGFR2 C17 antibody (Santa Cruz) or CN-14-3-3 adsorbed to protein A-Sepharose. Immunoprecipitates and pull-downs were washed in NP-40 buffer resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoprecipitates were immunoblotted with 4G10, anti-phospho-S779 FGFR2, anti-FGFR2 C17 (Santa Cruz), and EB1 while pull-downs were immunoblotted with anti-FGFR2 C17. Cleared cell lysates were also subjected to SDS-PAGE and immunoblotted with anti-phospho-Akt (Cell Signaling Technology), anti-phospho-extracellular signal-regulated kinase 1 and 2 (ERK1/2), anti-phospho-PLC-γ (Biosource), anti-phospho-GSK3α/β (Cell Signaling Technology), anti-actin (Chemicon), or anti-ERK1/2 (Promega) antibodies.

PI-3 kinase reporter assay. Ba/F3 cells stably expressing wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were electroporated with 20 μg of yellow fluorescent protein-pleckstrin homology (PH) domain of Akt1. After 24 h cells were factor and serum deprived for 8 h in RPMI medium containing 0.1% FCS prior to stimulation with 20 ng/ml FGF2 or IL-3 for various times. Cells were fixed (3.7% formaldehyde in PBS) and analyzed under a fluorescent confocal microscope.

Immunofluorescence. Ba/F3 cells stably expressing wt FGFR2 or FGFR2-S779G were factor and serum deprived for 4 h in RPMI medium containing 0.1% FCS prior to stimulation with 20 ng/ml FGF2 for the indicated times in the presence of heparin. Cells were then adhered onto poly-L-lysine-coated coverslips and fixed (3.7% formaldehyde in PBS). Cells were washed three times each with PBS and PBS-Tween, permeabilized with 0.1% Triton X-100, and incubated with blocking solution (1% bovine serum albumin) for 1 h at room temperature. Slides were probed with anti-FGFR2 antibody overnight at 4°C, washed in PBS-Tween, incubated with anti-rabbit antibody-Alexa488 (Invitrogen) and phalloidin-Alexa555 (Invitrogen) for 2 h at room temperature, washed, and analyzed by fluorescent confocal microscopy.

Cross-linking. Ba/F3 cells stably expressing wt FGFR2 or FGFR2-S779G were incubated with 20 ng/ml FGF at 4°C for 4 h. Cells were then left untreated or treated with BS3 cross-linker (Pierce) for 30 min, washed with PBS, and lysed. Cleared cell lysates were then subjected to SDS-PAGE and immunoblotted with anti-FGFR2 C17 antibody (Santa Cruz).

Protein stability assays. Ba/F3 cells stably expressing wt FGFR2 or FGFR2-S779G were seeded in RPMI medium supplemented with 10% FCS–10% WEHI 3B medium and 10 μg/ml cycloheximide (Sigma) in the presence or absence of 20 ng/ml FGF for up to 24 h. Following incubation, cells were lysed, and cleared lysates were subjected to SDS-PAGE and immunoblotted with anti-FGFR2 C17 antibody (Santa Cruz).

Predikin analysis. Predikin 2.0 analysis was performed essentially as described by Brinkworth et al. (3) using a Web-based interface (http://predikin.biosci.uq.edu.au/pkr/). Predikin analysis entailed analysis using both the kinase substrate database (KSD) and the substrate-determining residues (SDRs). SDRs are conserved amino acids, located in the catalytic domain of a serine/threonine kinase, which determine whether a protein is a likely substrate for the kinase. A database search identifies protein kinases with similar SDRs and, where possible, the phosphorylation sites at which they act. The phosphorylation sites are used to build a frequency matrix describing the predicted amino acid frequency at positions −3 to +3 for query kinases. The frequencies are converted to weights, which can be used to scan and score an input substrate sequence. For KSD analysis, a database query selects kinases of the same KSD family and their phosphorylation sites. The sites are used to build frequency and weight matrices that can be used to screen and score a substrate sequence.

RESULTS

S779 of FGFR2 is phosphorylated in response to FGF2 and binds the 14-3-3 proteins. A schematic representation of FGFR2 is shown in Fig. 1A. The cytoplasmic domain of FGFR2 contains seven known phosphorylation sites (Y463, Y583, Y585, Y653, Y654, Y730, and Y766), each of which is conserved across mammalian species in both FGFR2 and FGFR1. While some of these tyrosines have been linked to the regulation of specific signaling events, it has proved more difficult to functionally link individual tyrosine residues to specific biological responses (5, 22, 24, 35, 49, 60). We therefore considered the possibility that other nontyrosine motifs in the cytoplasmic domain of the FGFR may be important for regulating specific biological responses. We along with others have previously shown that the 14-3-3 family of proteins are able to bind specific phosphoserine residues in the cytoplasmic domains of cytokine and growth factor receptors (4, 17–19, 32, 39, 40, 52, 57, 62). The prototypic consensus binding motifs for the 14-3-3 proteins are R-S-X-S/$\underline{\text{T}}$-X-P (mode 1) and R-X-ψ-X-S/$\underline{\text{T}}$-X-P (mode 2) (where S/$\underline{\text{T}}$ is a phosphoserine/threonine, X is any amino acid, and ψ is a hydrophobic residue) (71). More recently, we along with others have identified 14-3-3 binding sites that do not strictly conform to these prototypic motifs (2, 62, 68). We identified one possible 14-3-3 binding site at S779 in FGFR2 (^{775}Q-Y-S-P-$\underline{\text{S}}$-Y-P^{781}, where $\underline{\text{S}}$ represents the putative phospho-S779) (Fig. 1A). Importantly, S779 lies distal to Y766 in the C-terminal lobe (Fig. 1A) and is conserved across mammalian species in both FGFR2 and FGFR1.

To determine whether the 14-3-3 proteins were able to directly interact with phospho-S779, we examined the ability of peptides encompassing the putative 14-3-3 binding site in FGFR2 to pull down purified recombinant 14-3-3ζ in vitro. As shown in Fig. 1B, the phospho-S779 peptide was able to precipitate recombinant 14-3-3ζ while the non-phospho-S779 peptide did not. Furthermore, the phospho-S779 peptide was able to precipitate 14-3-3 from Ba/F3 whole-cell lysates (Fig. 1B).

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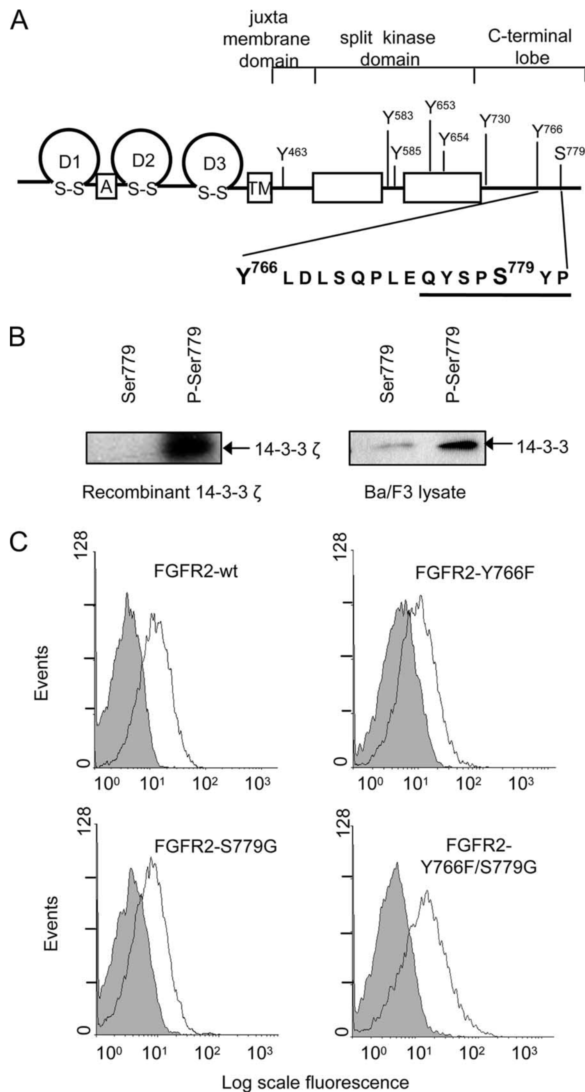

FIG. 1. Schematic representation of FGFR2 and the identification of a putative 14-3-3 binding site at S779. (A) Shown are immunoglobulin-like (Ig) domains (D1, D2, and D3), the acidic box (A), the transmembrane domain (TM), the juxtamembrane domain, the split tyrosine kinase domain, and the C-terminal lobe. Conserved cytoplasmic tyrosine autophosphorylation sites are indicated, and the putative 14-3-3 binding site surrounding S779 is underlined. (B) The ability of either non-phospho-S799 (S779) or phospho-S799 (P-S779) peptides encompassing S779 of FGFR2 to precipitate recombinant purified 14-3-3ζ and 14-3-3 from Ba/F3 lysates was examined using a pull-down approach. Pull-downs were subjected to SDS-PAGE and immunoblot analysis using anti-14-3-3 polyclonal antibody. Results are representative of at least two experiments. (C) Pools of transfected Ba/F3 cells stably expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were first stained with either a control (shaded profile) or FGFR2 primary antibodies (open profile), followed by FITC-conjugated anti-rabbit secondary antibodies, and cell surface expression of FGFR2 was analyzed by flow cytometry.

These results indicate that S779 of FGFR2 lies within a 14-3-3 binding site and could potentially bind the 14-3-3 proteins.

To test the possibility that S779 could bind the 14-3-3 proteins within the context of the full-length receptor in cells, we generated a construct for the expression of FGFR2-S779G. In addition, we also generated constructs in which the PLC-γ binding site at Y766 was mutated (FGFR2-Y766F) or both the PLC-γ and 14-3-3 binding sites were mutated (FGFR2-Y766F/S779G). We utilized the Ba/F3 cell system that has been widely used to examine FGFR signaling and functions (22, 26, 42, 59, 66). Importantly, Ba/F3 cells do not express endogenous FGFRs and therefore allow intracellular signaling and biological responses mediated by wt and mutant FGFRs to be characterized in an FGFR-null background. Pools of Ba/F3 cells stably expressing either wt or mutant FGFR2 were isolated. Analysis of surface receptor expression by flow cytometry indicated that wt and mutant FGFR2s were expressed at equivalent levels (Fig. 1C). We first examined the ability of 14-3-3 to interact with wt and mutant FGFR2s in a pull-down experiment using either Sepharose resin (control) or 14-3-3ζ–Sepharose. As shown in Fig. 2A, 14-3-3ζ–Sepharose was able to precipitate the wt FGFR2 while essentially no interaction with the Sepharose control was detected. However, while 14-3-3ζ–Sepharose was able to precipitate the Y766F mutant, interaction with the FGFR2-S779G and the FGFR2-Y766F/S779G mutants was reduced by more than sevenfold (Fig. 2A).

Although, the ability of 14-3-3ζ to precipitate FGFR2 did not appear to be regulated by FGF2 stimulation in the experiments shown in Fig. 2A, these experiments did not address whether endogenous 14-3-3 was able to associate with S779 of FGFR2 in response to ligand. We therefore examined the ability of FGFR2 to coimmunoprecipitate with 14-3-3 in response to FGF2. As shown in Fig. 2B, wt and FGFR2-Y766F coimmunoprecipitated with endogenous 14-3-3 in response to FGF2 stimulation, whereas no such interaction was detectable for the FGFR2-S779G and FGFR2-Y776F/S779G mutants. We therefore sought to determine whether S779 of FGFR2 was phosphorylated in response to FGF2. For these experiments, we generated anti-phospho-S779 phospho-specific polyclonal antibodies (described in Materials and Methods). These antibodies were shown to specifically recognize a phospho-S779 peptide but not a non-phospho-S779 peptide in dot blot experiments (Fig. 2C). As an additional control, we further showed that the anti-phospho-S779 phospho-specific polyclonal antibodies did not recognize a phosphoserine peptide derived from a different 14-3-3 binding site in the GM-CSF receptor (Fig. 2C). As shown in Fig. 2D, FGF2 stimulation of Ba/F3 cells expressing wt FGFR2 resulted in a rapid increase in S779 phosphorylation. Importantly, no phosphorylation was observed for the FGFR2-S779G mutant, further demonstrating the specificity of the anti-phospho-S779 antibodies.

We further examined whether mutation of S779 had an impact on FGFR2 tyrosine phosphorylation. As shown in Fig. 3A, equivalent levels of receptor tyrosine phosphorylation were observed in Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G following FGF2 stimulation. To further confirm that there was no overall defect on receptor tyrosine phosphorylation for each of the mutants, we also generated BALB/c 3T3 cell lines stably expressing wt FGFR2, FGFR2-S779G, FGFR2-Y766F, and FGFR2-Y766F/S779G. As shown in Fig. 3B, no detectable difference was observed between the wt and mutant FGFR2s in terms of receptor tyrosine phosphorylation. We then compared the kinetics of S779 phosphorylation with FGFR2 tyrosine phosphorylation. As shown in Fig. 3C, the induction of

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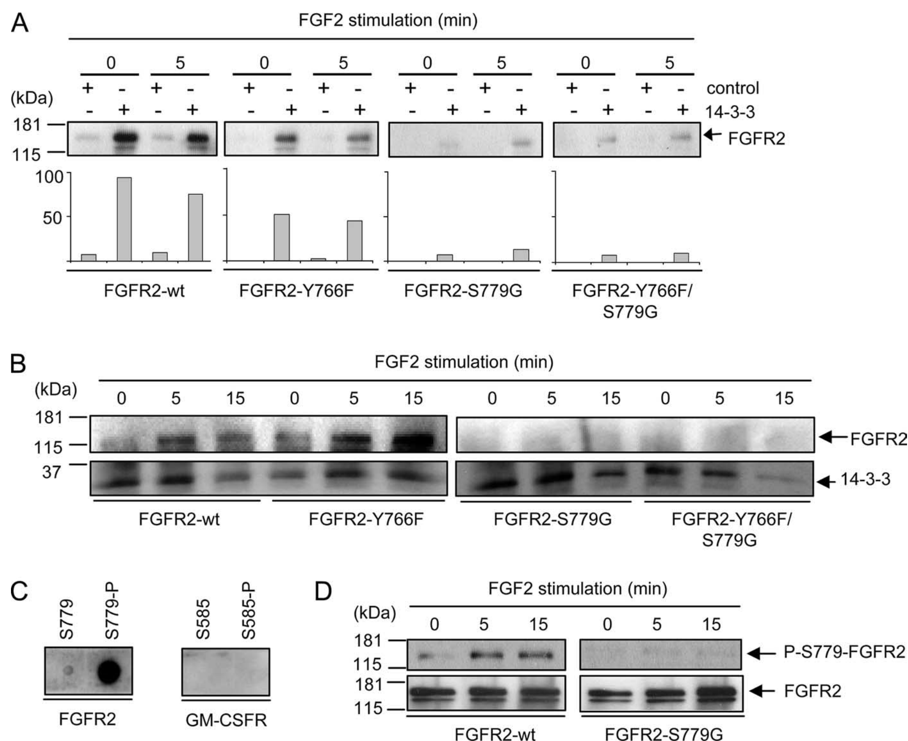

FIG. 2. S779 of FGFR2 is phosphorylated in response to FGF2 and binds the 14-3-3 family of proteins. Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were starved in medium containing 0.1% FCS for 4 h and then stimulated with 20 ng/ml FGF2 for the indicated times. (A) Cells were then lysed and pull-downs performed using either Sepharose alone (control) or CnBr-coupled 14-3-3ζ–Sepharose. Pull-downs were resolved by SDS-PAGE and immunoblotted with anti-FGFR2 polyclonal antibody. (B) In addition, cells were also stimulated as above, and 14-3-3 was immunoprecipitated. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-FGFR2 polyclonal antibody. (C) Specificity of anti-phospho-S779 phospho-specific antibodies was confirmed in dot blot assays. A total of 4 μg of non-phospho-S799 (S779) or phospho-S799 (P-S779) peptide encompassing the 14-3-3 binding site at S779 of FGFR2 (left panel) or non-phospho-S585 (S585) or phospho-S585 (P-S585) control peptide encompassing a different 14-3-3 binding site in the GM-CSF receptor (GM-CSFR) was blotted onto nitrocellulose and incubated with anti-phospho-S779 antibody followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody. (D) Ba/F3 cells were stimulated with FGF2, and FGFR2 was immunoprecipitated with anti-FGFR2 polyclonal antibody. Immunoprecipitates were then subjected to SDS-PAGE and immunoblot analysis using the anti-phospho-S779 phospho-specific antibodies.

both S779 phosphorylation and tyrosine phosphorylation of FGFR2 both occurred within 5 min; however, while receptor tyrosine phosphorylation was clearly transient, S779 phosphorylation was sustained and persisted for up to 4 h. It is also important to note that lack of S779 phosphorylation and 14-3-3 binding in the S779G mutant did not affect receptor tyrosine phosphorylation induction or kinetics (Fig. 3C).

Induction of S779 phosphorylation by the protein kinase C (PKC) family of kinases. As an initial step toward the identification of kinases involved in S779 phosphorylation, we performed Predikin analysis, which is a predictive algorithm that can be used to identify substrates for known kinases (3). Predikin utilizes data from available crystal structures, molecular modeling, and the sequence analysis of kinases and substrates to predict the requirements for binding of a heptapeptide substrate motif to a specific kinase. In silico analysis using Predikin, version 2.0 (http://predikin.biosci.uq.edu.au/pkr/), that incorporated both the SDRs and the KSD resulted in the identification of a number of kinases with scores of >65 (out of 100), indicating a potential to phosphorylate S779. These kinases included PKC (score, 76.89), IκB kinase (IKK; score, 67.42), Akt (also known as PKB; score, 72.87), and GSK3 (score, 84.23). We then examined the possible involvement of these candidate kinases identified by Predikin by examining the pharmacological profile of S779 phosphorylation using inhibitors for PI 3-kinase (LY294002 and Wortmannin), mTOR (rapamycin), GSK3 (VIII), IKK (BAY11-7082), PKA (H89), JAK tyrosine kinases (JAKI), and the FGFR2-specific tyrosine kinase inhibitor SU5402. While Predikin reported PKC as a potential S779 kinase, Davies et al. have shown very clearly that essentially all widely used PKC inhibitors have very poor selectivity (8). We therefore examined the ability of a widely used specific activator of PKC, phorbol 12-myristate 13-acetate (PMA), to induce ligand-independent S779 phosphorylation.

As shown in Fig. 3D, neither H89, Wortmannin, rapamycin, LY294002, JAKI, GSK3β1, or BAY11-7082 was able to block (or reduce) S779 phosphorylation in response to FGF2. Thus, our data exclude a role for these kinases (or their downstream targets) in phosphorylating S779. However, we observed an increase in S779 phosphorylation following PMA stimulation, suggesting the involvement of the PKC family of kinases. Time course experiments showed that the induction of S779 phosphorylation by PMA demonstrated similar kinetics to those observed following FGF2 stimulation (data not shown). These

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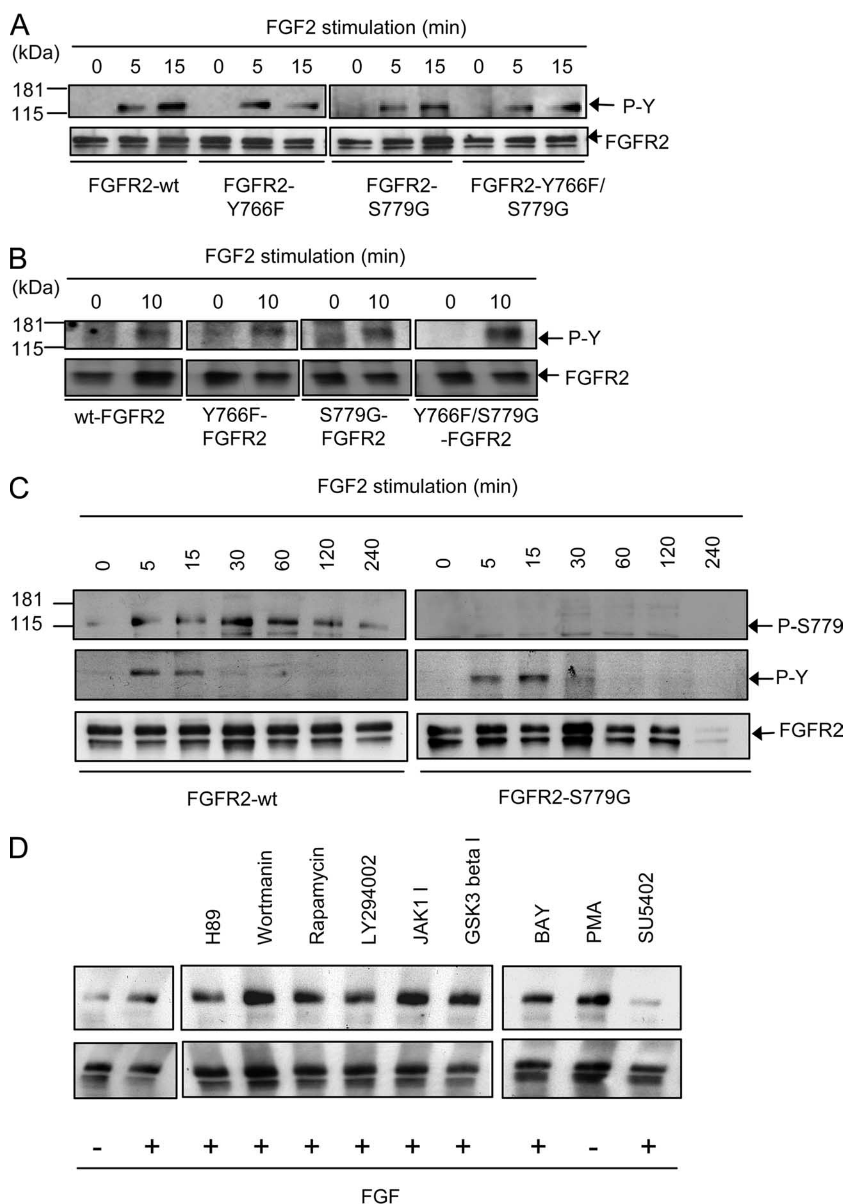

FIG. 3. The regulation of S779 and receptor tyrosine phosphorylation by wt and mutant FGFR2s. (A and C) Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were starved in medium containing 0.1% FCS for 4 h and then stimulated with 20 ng/ml FGF2 for the indicated times. Cells were lysed, FGFR2 was immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis with either the 4G10 antiphosphotyrosine monoclonal antibody (P-Y), anti-phospho-S779, or anti-FGFR2 polyclonal antibody. (B) BALB/c 3T3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were starved for 24 h and then stimulated with FGF2 as above. FGFR2 immunoprecipitates were then subjected to immunoblot analysis as in panel A. (D) wt FGFR2 cells were starved as described for panel A with the inclusion of indicated inhibitors 30 min prior to stimulation with FGF or followed by stimulation with PMA. FGFR2 immunoprecipitates were then subjected to immunoblot analysis as in panel A. Results shown are representative of at least two experiments.

results suggest a possible role for the PKC family of kinases in regulating S779 phosphorylation.

FGFR2 regulates specific intracellular signaling pathways via S779. The regulation of S779 phosphorylation and 14-3-3 recruitment in response to FGF2 stimulation raised the possibility that these events may be important in mediating FGFR2 intracellular signaling. We therefore examined the possible role of S779 in regulating two major FGF signaling pathways: namely the Ras/MAP kinase pathway (by examining ERK phosphorylation) and the PI 3-kinase pathway (by examining Akt phosphorylation). As shown in Fig. 4A, B, and C, there was no apparent difference in the ability of FGF2 to promote Akt and ERK phosphorylation in Ba/F3 cells expressing either the wt FGFR2 or the FGFR2-Y766F mutant. However, we observed that Akt phosphorylation was significantly reduced by more than 60% in cells expressing both the FGFR2-S779G mutant (Fig. 4A and B) and the FGFR2-Y766F/S779G mutant (Fig. 4A and B) compared to cells expressing wt FGFR2.

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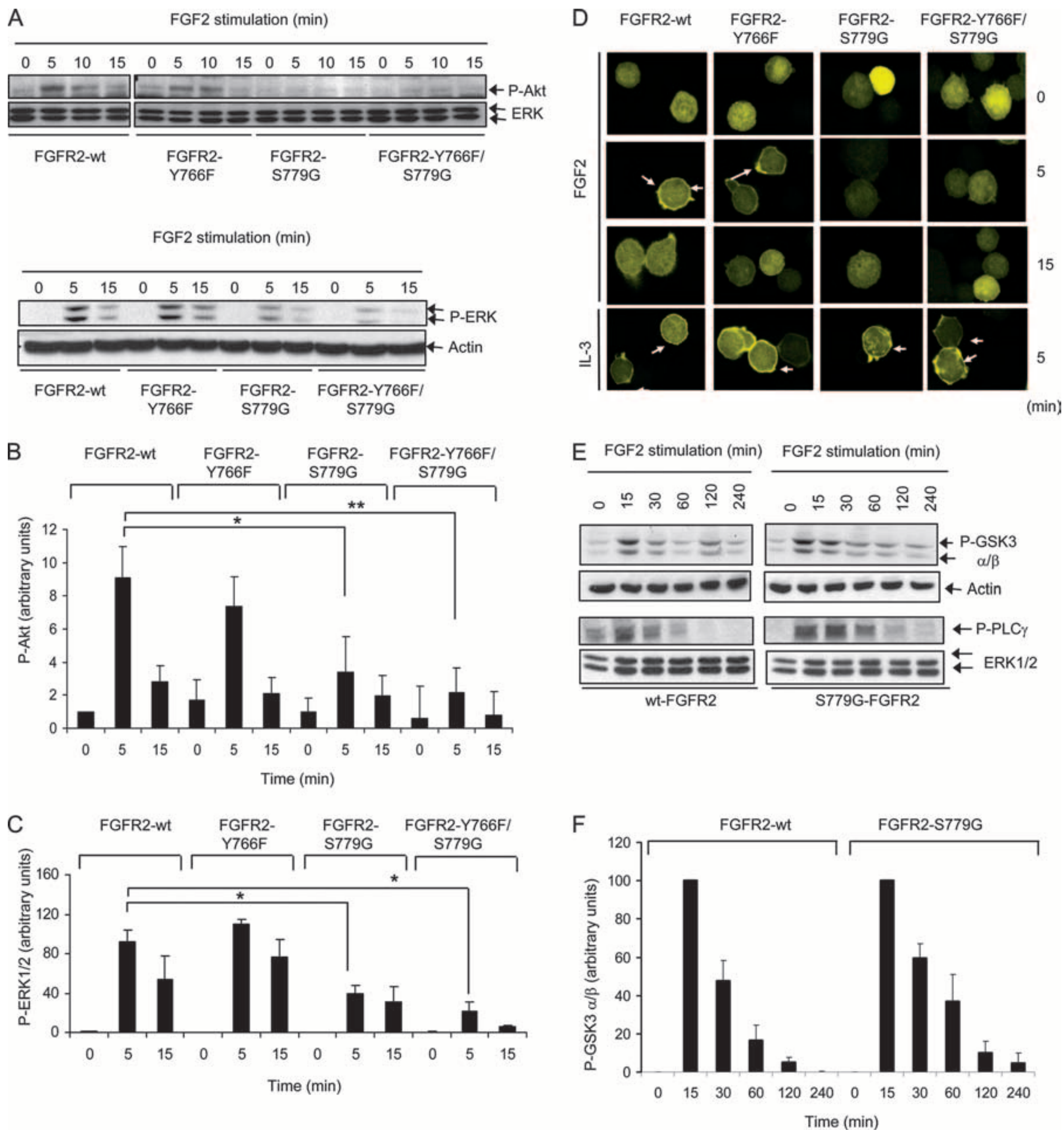

FIG. 4. S779 of FGFR2 is required for signaling via the PI 3-kinase and Ras/MAP kinase pathways. Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were starved and stimulated with FGF2 as described in the legend to Fig. 2. Cell lysates were then subjected to SDS-PAGE and immunoblotted with anti-phospho-Akt, anti-phospho-ERK, and anti-ERK1/2 polyclonal antibody or antiactin polyclonal antibody (A). Quantitation of the phospho-Akt (B) or phospho-ERK1/2 (C) signals from two independent experiments is shown (*, $P < 0.02$; **, $P < 0.008$). Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were electroporated with a construct for the expression of a fusion protein containing GFP and the PH domain of Akt1 (PH-Akt1-GFP). After 24 h, cells were starved and stimulated with FGF2 or IL-3 (positive control) for the indicated times as described in the legend to Fig. 2. Cells were fixed, and the localization of PH-Akt1-GFP was analyzed by confocal fluorescence microscopy (D). Arrows indicate membrane localization of PH-Akt1-GFP. (E) The regulation of GSK3 and PLC-γ phosphorylation in response to FGF2 was determined as described for panel A. Quantitation of phospho-GSK3 signals from two independent experiments is shown. Results are representative of at least two experiments.

Similarly, compared to cells expressing wt FGFR2, ERK phosphorylation was reduced by 58% in cells expressing the FGFR2-S779G mutant and by 77% in cells expressing the FGFR2-Y766F/S779G mutant (Fig. 4A and C).

To further investigate the role of S779 in PI 3-kinase signaling, we examined the ability of FGF2 to induce the membrane localization of a fusion protein containing the PH domain of Akt1 and green fluorescent protein ([GFP] PH-Akt1-GFP). The PH-Akt1-GFP binds PI (3, 4, 5) triphosphate (PIP_3) in the plasma membrane following PI 3-kinase activation, and this localization can be observed by confocal fluorescence microscopy (16). Ba/F3 cells expressing either wt or mutant FGFR2 were transfected with a construct for the expression of PH-Akt1-GFP, starved of factor, and then stimulated with either

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IL-3 (control) or FGF2. As shown in Fig. 4D, FGF2 stimulation of cells expressing wt FGFR2 or FGFR2-Y766F resulted in the translocation of PH-Akt1-GFP to the plasma membrane at 5 min (Fig. 4D, arrows) and subsequent redistribution to the cytoplasm at 15 min (Fig. 4D), indicating that PIP_3 production in response to FGF2 was both rapid and transient. However, no such localization was observed for either the FGFR2-S779G or the FGFR2-Y766F/S779G mutants following FGF2 stimulation at either 5 or 15 min, indicating a defect in the activation of PI 3-kinase and the generation of PIP_3.

While the results shown in Fig. 4A, B, and D indicated that the FGFR2-S779G mutant was defective in PI 3-kinase signaling in response to FGF2, we did not observe any significant defect in the phosphorylation of the downstream PI 3-kinase target, GSK3 (Fig. 4E and F). These results would suggest that GSK3 phosphorylation in response to FGF2 may occur via a PI 3-kinase- and Akt-independent pathway in Ba/F3 cells. In fact, others have shown that GSK3 can be phosphorylated by PKA and PKC independently of PI 3-kinase activity (11, 67). Alternatively, it is also possible that the residual Akt phosphorylation observed for the S779G mutant in Fig. 4A was sufficient for phosphorylation of GSK3. In addition, no difference in Y783 phosphorylation of PLC-γ following FGF2 stimulation was observed between cells expressing the wt and mutant FGFR2s (Fig. 4E).

FRS2 has been shown to play an important role in the ability of the FGFRs to promote both PI 3-kinase signaling and Ras-MAP kinase signaling (20, 23). However, we were unable to detect FRS2 phosphorylation in response to FGF2 in Ba/F3 cells expressing either wt or mutant receptors (data not shown). Despite the lack of detectable FRS2 phosphorylation, wt FGFR2 was clearly able to promote PI 3-kinase and Ras-MAP kinase signaling (Fig. 4) as well as proliferation and survival of Ba/F3 cells (see below). In this respect, it is noteworthy that others have observed FGFR2 intracellular signaling and biological responses in the absence of detectable FRS2 tyrosine phosphorylation (21, 27).

We then sought to determine whether the observed defects in receptor signaling for the FGFR2-S779G mutant were due to defects in receptor dimerization, stability, or internalization. To examine receptor dimerization, we utilized the BS3 cross-linker that allows the amine-reactive *N*-hydroxysulfosuccinimide ester at each end of an 8-carbon spacer arm to form stable bonds, with primary amines such as side chains of lysine residues (61). FGF2 stimulation of cells expressing the wt FGFR2 in the presence of the BS3 chemical cross-linker resulted in a mobility shift consistent with the formation of ligand-induced higher-order oligomeric FGFR2 complexes (Fig. 5A, asterisk). Importantly, no significant difference in the formation of BS3-cross-linked receptor oligomers was observed between the wt and the FGFR2-S779G receptors (Fig. 5A). These results indicate that the S779G mutant was not defective in ligand-induced dimerization and are consistent with the data shown in Fig. 3 demonstrating that the S779G mutant was not defective in mediating ligand-induced receptor tyrosine phosphorylation.

We then compared the half-life of the FGFR2-S779G mutant to the wt receptor by examining the loss of FGFR2 in the presence of protein synthesis inhibitor, cycloheximide. As shown in Fig. 5B, protein stability of the wt FGFR2 and the FGFR2-S779G mutant was similar both in the absence of FGF2 (steady-state half-life, 9.3 $\pm$ 2.2 and 9.6 $\pm$ 0.5 h, respectively) and following FGF2 stimulation (ligand-induced half-life, 8.9 $\pm$ 1.7 and 8.1 $\pm$ 0.2 h, respectively).

Finally, we examined whether receptor internalization following FGF2 stimulation was defective for the S779G mutant. Using confocal fluorescence microscopy to localize FGFR2 together with a phalloidin counterstain to decorate cortical actin at the plasma membrane, we show that both the wt and the FGFR2-S779G mutant are internalized following FGF2 stimulation (Fig. 5C). Collectively, the results shown in Fig. 5 indicate that the observed defects in receptor signaling for the FGFR2-S779G mutant were not simply due to defects in either receptor dimerization, stability, or internalization.

FGFR2 regulates cell proliferation and survival via S779. To determine the functional consequences of S779 phosphorylation and 14-3-3 signaling, we first examined the ability of FGF2 to promote the growth and viability of Ba/F3 cells expressing wt and mutant FGFR2s using the colorimetric MTS reduction assay. While FGF2 promoted a dose-dependent increase in MTS signal in cells expressing the wt and the FGFR2-Y766F mutant, this response was markedly reduced in the FGFR2-S779G and FGFR2-Y766F/S779G mutants (Fig. 6A). We further examined whether this functional defect in the FGFR2-S779G mutant also occurred in BALB/c 3T3 cells stably transfected with wt and mutant FGFR2s in the MTS assay. While FCS provided a potent mitogenic signal in all BALB/c 3T3 cell lines (positive control), cells expressing the FGFR2-S779G and the FGFR2-Y766F/S779G mutants demonstrated significantly reduced responses to FGF2 compared to cells expressing wt FGFR2 (Fig. 6B).

We then examined whether the defect observed in the MTS assay for the FGFR2-S779G mutant was specifically due to inability of FGF2 to promote cell survival. First, we examined cell death by quantifying the reduction in the number of viable cells containing $\geq$2N DNA (i.e., the sub-G_0/G_1 population) using propidium iodide staining and flow cytometry analysis. FGF2 was able to promote increased viability (as determined by $\geq$2N DNA content) in cells expressing wt FGFR2 and the FGFR2-Y766F mutant; however, no such response was observed in cells expressing the FGFR2-S779G or the FGFR2-Y766F/S779G mutants (Fig. 7A). To further determine whether the mode of cell death involved apoptosis, we specifically examined the loss of mitochondrial membrane potential using the fluorometric dye TMRE (15, 65). In the absence of FGF2, no TMRE retention was observed in cells expressing the wt FGFR2, indicating a loss of mitochondrial membrane potential associated with apoptosis. Although FGF2 was able to promote TMRE retention in cells expressing the wt FGFR2 and the FGFR2-Y766F (Fig. 7B), TMRE retention was significantly reduced in cells expressing either the FGFR2-S779G or the FGFR2-Y766F/S779G mutants (Fig. 7B), indicating that S779 of FGFR2 was critical in specifically regulating cell survival in response to FGF2.

We also examined the ability of the wt and mutant FGFR2s to promote cell proliferation by examining S phase entry using BrdU incorporation in response to FGF2. While FGF2 was able to promote the S phase entry of Ba/F3 cells expressing either the wt FGFR2 or the FGFR2-Y766F, S phase entry of the FGFR2-S779G and the FGFR2-Y766F/S779G mutants

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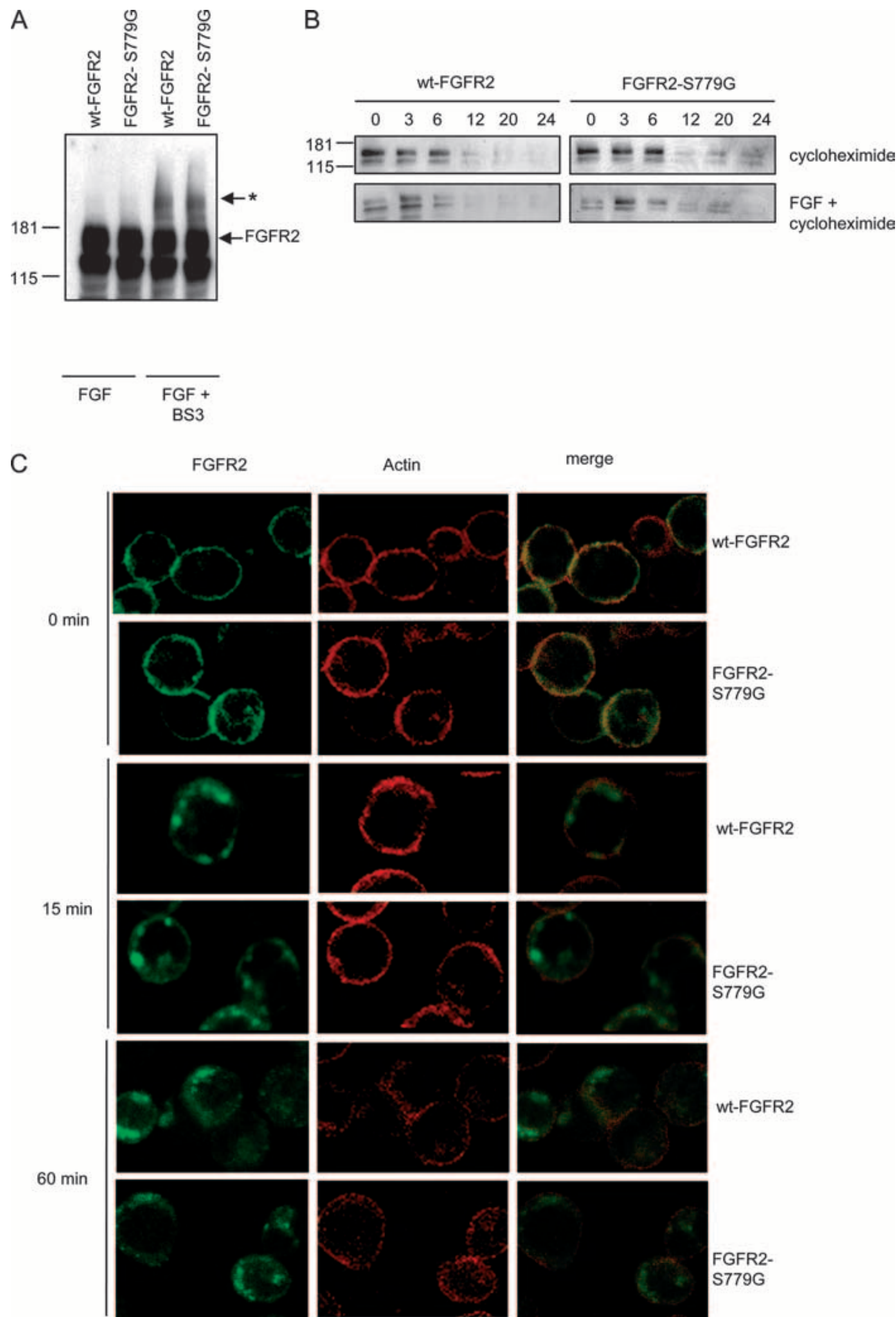

FIG. 5. Receptor dimerization, stability, and internalization are not affected in the FGFR2-S779G mutant. (A) Ba/F3 cells expressing either wt FGFR2 or FGFR2-S779G were incubated in the presence of FGF for 4 h and then left untreated or treated with BS3 cross-linker, lysed, and subjected to SDS-PAGE and immunoblotting with anti-FGFR2 polyclonal antibody. (B) Ba/F3 cells expressing either wt FGFR2 or FGFR2-S779G were plated in medium containing 10% fetal calf serum, 10% WEHI, and 10 μg/ml cycloheximide in the presence or absence of 20 ng/ml FGF2 for up to 24 h. Cells were then lysed and subjected to SDS-PAGE and immunoblotted with anti-FGFR2 polyclonal antibody. (C) Ba/F3 cells expressing either wt FGFR2 or FGFR2-S779G were starved in medium containing 0.1% FCS for 4 h prior to stimulation with 20 ng/ml FGF for 0, 15, and 60 min. Cells were fixed and stained with anti-FGFR2 polyclonal antibody (green) and costained with phalloidin (red). Results are representative of at least two experiments.

was significantly reduced (Fig. 8A). We further examined this defect in cell proliferation by analyzing the cell cycle distribution using propidium iodide staining of fixed cells followed by flow cytometry analysis. Cells expressing the wt FGFR2 and the FGFR2-Y766F mutant and growing asynchronously in FGF2 demonstrated similar cell cycle distributions. However, a significant reduction in the ability of FGF2 to promote entry into S phase in cells expressing the FGFR2-S779G and the FGFR2-Y766F/S779G mutants was observed (Fig. 8B). Together, our data demonstrate an important functional role for

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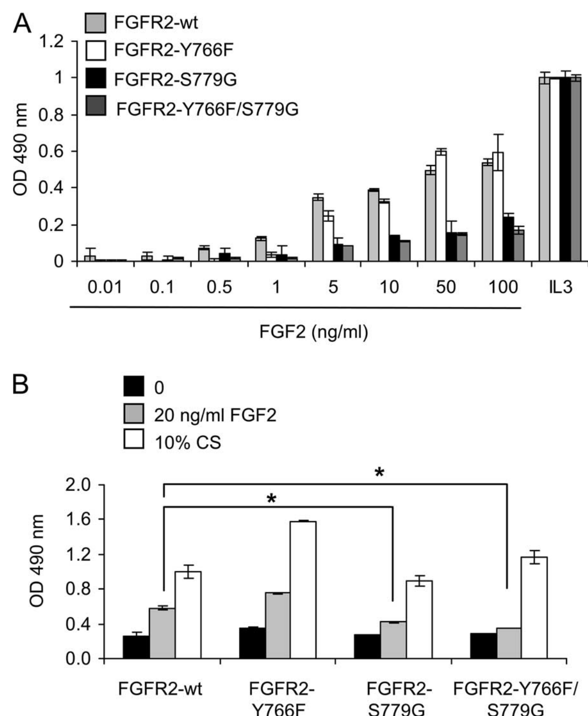

FIG. 6. S779 of FGFR2 promotes cell viability in response to FGF2. (A) Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were plated in medium containing 0.1% FCS and the indicated concentrations of FGF2. IL-3 was used as a positive control. After 24 h, the colorimetric MTS reduction assay was performed according to the manufacturer's instructions, and absorbance was measured at 490 nm. (B) In addition, the response of BALB/c 3T3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G was also examined using the MTS assay. Cells were plated in medium containing 10% BIT in the presence or absence of FGF2. FCS (10%) was used as a positive control. Cellular responses were measured after 3 days using the MTS assay. All results shown are representative of at least two experiments. Error bars represent standard deviations. $*$, $P < 6 \times 10^{-5}$.

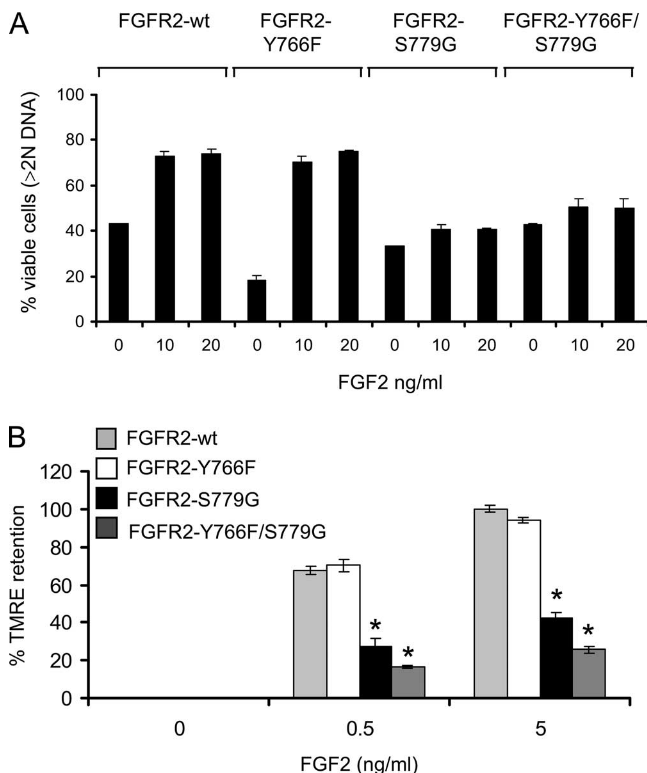

FIG. 7. S779 of FGFR2 regulates cell survival in response to FGF2. (A) Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were plated in medium containing 0.1% FCS in the presence or absence of 20 ng/ml FGF2. After 24 h, the cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. The percentage of viable cells (>2N DNA) is shown. (B) Cells were plated as above in the indicated concentrations of FGF2 and after 24 h were stained with TMRE and analyzed by flow cytometry. Shown is the percentage of cells demonstrating TMRE retention indicating intact mitochondrial potential. All results shown are representative of at least three experiments. Error bars represent standard deviations ($*$, $P < 0.05$).

S779 of FGFR2 and 14-3-3 signaling in regulating FGF2-mediated intracellular signaling and cellular responses.

DISCUSSION

The ability of phosphotyrosine docking sites in the cytoplasmic tails of growth factor receptors to bind SH2 and PTB domain proteins represents an important paradigm by which intracellular signaling and cellular responses are controlled. We now show that in addition to phosphotyrosine residues, a specific phosphoserine residue in the cytoplasmic domain of FGFR2 functions as a docking site for the 14-3-3 family of phosphoserine-binding proteins to regulate intracellular signaling pathways and biological responses. We have identified a conserved serine in the C-terminal lobe of FGFR2 (S779) that is phosphorylated in response to FGF2 stimulation and binds the 14-3-3 proteins (Fig. 2). Using the Ba/F3 model system to examine FGF2-mediated signaling and cellular responses (22, 26, 42, 59, 66), we have shown that a FGFR2-S779G mutant was not only unable to bind the 14-3-3 proteins but also demonstrated reduced Akt and ERK phosphorylation in response to ligand (Fig. 2 and 4). These results were in contrast to the FGFR2-Y766F mutant (that has been previously shown to be defective in PLC-γ signaling), which showed no defect in Akt or ERK phosphorylation (Fig. 4). Significantly, the FGFR2-S779G mutant that showed defective PI 3-kinase and Ras/MAP kinase signaling was also unable to support the survival or proliferation of Ba/F3 cells in response to FGF2 (Fig. 6, 7, and 8). No difference in FGFR2 tyrosine phosphorylation was observed between the wt and any of the mutant receptors, indicating that the inability of the FGFR2-S779G mutant to promote cell viability and proliferation was not simply due to an overall defect in receptor activation (Fig. 3). Furthermore, the inability of the FGFR2-S779G mutant to promote cell survival and proliferation was also simply not due to a global defect in the activation of all downstream signaling pathways. The defect in the ability of the FGFR2-S779G mutant to regulate cell survival and proliferation compared to the wt FGFR2 was not due to either (i) reduced ligand-induced receptor dimerization, (ii) altered protein stability, or (iii) reduced receptor internalization (Fig. 5). Rather, our results show that only specific FGFR2 signaling outputs appeared to be affected by the S779G mutation. This was evident from the ability of the FGFR2-S779G mutant to normally regulate GSK3 and PLC-γ

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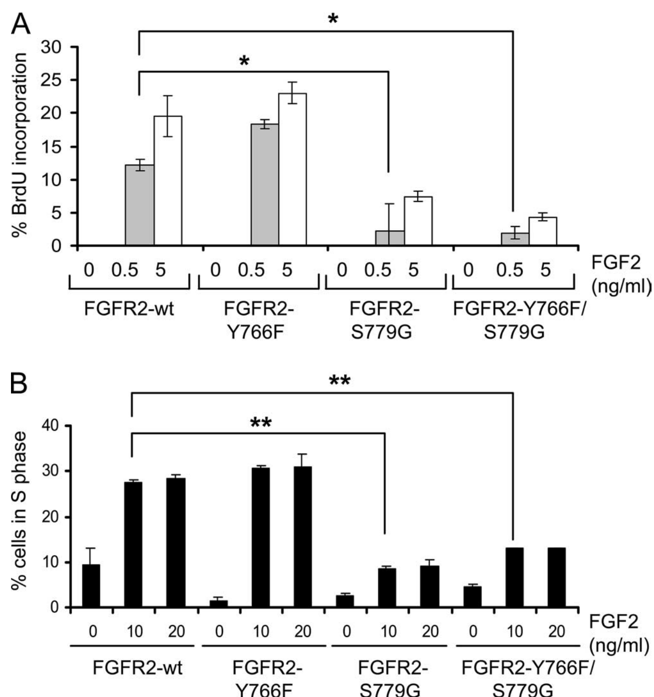

FIG. 8. S779 of FGFR2 regulates cell proliferation in response to FGF2. (A) Ba/F3 cells expressing either wt FGFR2, FGFR2-Y766F, FGFR2-S779G, or FGFR2-Y766F/S779G were plated in medium containing 0.1% FCS in either 0, 0.5, or 5 ng/ml FGF2. After 24 h, cells were pulsed with BrdU for 3 h, fixed, and stained with anti-BrdU-FITC monoclonal antibody, following which the cells were analyzed for BrdU incorporation by flow cytometry. (B) Cells were plated as above and after 24 h were fixed and stained with propidium iodide. Cell cycle distribution was then determined by flow cytometry. The percentage of cells in S phase was quantified from three experiments, and the results are shown. Error bars represent standard deviations (*, $P < 0.05$).

phosphorylation while other signaling events such as the phosphorylation of Akt and ERK were clearly disrupted (Fig. 4). Together, these results identify a new FGF signaling pathway in which phosphorylation of S779 in the cytoplasmic domain of FGFR2 provides a docking site for the 14-3-3 proteins and couples the activated receptor to specific signaling pathways biological responses.

In terms of the kinase that phosphorylates S779, it is known that the recognition motifs for some AGC-family serine/threonine kinases include basic residues N-terminal to the phosphoserine and therefore partially overlap with the prototypic mode 1 and mode 2 14-3-3 consensus binding motifs (R-S-X-S/T-X-P and R-X-ψ-X-S/T-X-P [for underlining, see Results]) (71). Thus, a number of AGC kinases, including Akt and PKA, have been shown to phosphorylate 14-3-3 binding sites in diverse proteins (1, 30). In the case of the GM-CSF and IL-3 receptors, we have shown a role for PKA in phosphorylating S585 of the βc subunit (19). However, the QYSPSYP motif in FGFR2 does not strictly conform to classic mode 1 or mode 2 recognition sequences and does not constitute a consensus PKA phosphorylation site (47). Nevertheless, the S779 motif in FGFR2 resembles the 14-3-3 binding sites identified in other proteins such as the mouse βc subunit of the GM-CSF receptor (^{600}QSHSLP^{605}) (our unpublished data), p53 (^{380}QSTpSRH^{385}), and AMP-activated kinase family QSK (^{159}QLIKTWC^{165}) (2, 68). Thus, in order to identify candidate kinases involved in S779 phosphorylation, we performed Predikin analysis (3). This analysis revealed PKC, IKK, Akt, and GSK3 as potential hits. We then examined the pharmacological profile of these kinases in terms of S779 phosphorylation. We showed that inhibition of IKK, Akt, and GSK3 did not reduce S779 phosphorylation (Fig. 3D). However, we also showed that pharmacological activation of PKC with PMA resulted in the ligand-independent phosphorylation of S779. Thus, our results suggest a role for the PKC family of kinases in phosphorylating S779. Others have shown important roles for PKC in FGF-mediated biological responses. For example, a role for PKC-ε in the FGF2-mediated survival of small cell lung cancer cells has been suggested (43). In addition, while EGF stimulation of the PC12 pheochromocytoma cells line normally promotes proliferation, costimulation of these cells with EGF and PMA converts the EGF-proliferative response into an FGF-like differentiation response, and the cells develop neurite-like outgrowths (53). Furthermore, small interfering RNA of PKC-δ reduced FGF-mediated PC12 differentiation, suggesting an important role for PKC-δ in mediating at least some specific biological responses (53). While it is not clear which of the more than 10 members of the PKC family of kinases is important for S779 phosphorylation, our results show an important role for S779 signaling in regulating specific FGF biological responses.

In the same manner as phosphotyrosine residues are able to promote the reversible assembly of signaling complexes via the binding of SH2 and PTB domain proteins, phosphoserine/threonine residues have been shown to act as docking sites for the 14-3-3 proteins (4, 33, 39, 40, 52, 57). However, while the binding of SH2 and PTB domain proteins to phosphotyrosine residues in the cytoplasmic domains of growth factor and cytokine receptors has been widely demonstrated to allow the dynamic regulation of intracellular signaling pathways, it is only very recently that the phosphorylation of receptor serine residues and the binding of the 14-3-3 proteins have been reported. For example, our earlier studies have shown that S585 in the cytoplasmic domain of the βc subunit in the GM-CSF and IL-3 receptors is phosphorylated in response to ligand and binds the 14-3-3 proteins (19, 62). Importantly, these earlier studies showed that S585 phosphorylation and 14-3-3 binding were required for the regulation of PI 3-kinase signaling and cell survival (19). Subsequent studies have shown that S585 phosphorylation of βc couples to distinct signaling pathways and biological responses compared to βc tyrosine phosphorylation, providing a mechanistic explanation for how GM-CSF and IL-3 are able to regulate pleiotropic (or diverse) biological responses (17, 18). Thus, our studies examining growth factor and cytokine receptors now establish a new mode of signaling involving receptor serine phosphorylation and 14-3-3 signaling that performs essential roles in mediating specific cellular responses.

Other examples have been reported in which receptor serine phosphorylation and 14-3-3 binding have been shown to regulate intracellular signaling pathways. For example, a 14-3-3 binding site in the C-terminal lobe of the IGF-1R has been identified (4). Subsequent studies have examined the possible functional roles of specific tyrosine and serine residues in the cytoplasmic domain of IGF-1R by examining the ability of wt

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TABLE 1. Identification of putative conserved phosphotyrosine/serine motifs in diverse cell surface receptors

Name	Conserved motif		Accession no.
	Start position[a]	Sequence[b]	
FGFR2 (Bek)	763	NEE**Y**LLSQPLE......................QYSP**S**YP	P21802
GM-CSF/IL-3/IL-5 receptor βc subunit[c]	590	NGP**Y**LGPP..........................HSR**S**LP	NP_000386
FGFR1(Flg)	763	NQE**Y**LDLSIPLD.....................QYSP**S**FP	P11362
ErbB4	1239	NPD**Y**..............................WNH**S**LP	NP_005226
Low-density lipoprotein receptor-related protein 1	4470	NPT**Y**KMYEGGEPDDVGGLLDADFALDPD....KPTNF**T**NP	Q07954
Low-density lipoprotein receptor	802	NPV**Y**QKTTEDEVHICHN................QDGY**S**YP	AAF24515
Very-low-density lipoprotein receptor	834	NPV**Y**LKTTEEDLSIDIG................RHSA**S**VG	NP_003374
Integrin beta 1 (fibronectin receptor)	780	NPI**Y**............................KSAVT**T**VV	NP_002202
Integrin beta 3 (platelet glycoprotein IIIa)	770	NPL**Y**KEA..........................TSTF**T**NI	NP_000203
Integrin beta-6	759	NPL**Y**R............................GSTS**T**FK	A37057
Integrin beta-7	775	NPL**Y**KS...........................AITT**T**IN	P26010
ErbB3	972	PPR**Y**LVI...........................KRE**S**GP	P21860
FLT3 receptor tyrosine kinase	916	EEI**Y**IIMQSCWAFDS..................RKRP**S**FP	CAA81393
Vascular endothelial growth factor receptor 2	1220	ISQ**Y**LQNS..........................KRK**S**RP	AAC16450
Granulocyte colony stimulating factor receptor	764	PGH**Y**L.............................RDS**T**QP	Q99062
IL-6 receptor beta chain (glycoprotein 130)	764	HSG**Y**RHQVPSVQVFS..................RSES**T**QP	P40189
TrkB tyrosine kinase (BDNF/NT-3 receptor)	752	QPW**Y**QLSNNEVIECITQGRVL............QRPR**T**CP	Q16620
Platelet-derived growth factor receptor alpha	608	GTA**Y**.............................GLSR**S**QP	P16234
c-Met	1023	QVQ**Y**PLTDMSPILTSG.................DSDI**S**SP	P08581
Macrophage-stimulating protein receptor (p185-Ron)	1357	PAT**Y**MNLGPSTSHEMNVRPEQPQFSPMPGNVRRPRPL**S**EP	Q04912
IGF-1R	1278	SFY**Y**SEENKLPEPEELDLEPENMESVPLD..PSASSS**S**LP	NP_000866

[a] Residue number.
[b] The tyrosine and serine residues in boldface in each motif are conserved between at least three species including, human, mouse, and rat.
[c] References 15 and 16.

and mutant receptors to promote intracellular signaling as well as cell proliferation and survival in the murine 32D factor-dependent cell line (48). These studies showed that mutating key serine residues within the 14-3-3 binding site of IGF-1R reduced the ability of IGF-1 to promote mitochondrial translocation of Raf1, the phosphorylation of BAD, and the survival of 32D cells (48). Thus, the dual ability of receptor serine phosphorylation and 14-3-3 signaling together with receptor tyrosine phosphorylation and SH2 and PTB domain protein signaling may be essential for promoting diversity in signaling and specificity in cellular responses.

The identification of a specific serine phosphorylation site in FGFR2 that regulates a 14-3-3 signaling pathway leading to cell proliferation and survival has important implications in terms of how the FGFs are able to regulate pleiotropic biological responses. Since the discovery of the prototypic family members, FGF1 and FGF2, a large number of studies have shown that the FGFs coordinately regulate diverse cellular responses including cell survival, proliferation, and differentiation that underpin key processes involved in development, hematopoiesis, angiogenesis, and tissue repair (7, 9, 50, 64). In fact, the use of gene knockout approaches in mice has demonstrated that FGFs and their cognate receptors are able to dictate multiple and diverse cell fates, providing an extensive picture of their pleiotropic functions (9). However, the underlying mechanisms by which the FGFRs regulate these diverse functions remain largely undefined. While a number of reports have examined the link between individual FGFR tyrosine residues and specific FGF-mediated biological responses (5, 6, 12, 22, 25, 35, 38, 44, 51), it is still not known how the FGFs are able to specify different cellular outcomes. While there is clear evidence that specific tyrosine residues in some RTKs provide qualitatively unique signals that mediate defined cellular responses (46), some studies have reported redundancy in receptor tyrosine phosphorylation and have suggested that RTKs may generate generic tyrosine signals that are interpreted in quantitatively different manners to promote specific cellular outcomes (10, 31, 56, 63). Our studies now provide a new and alternative mechanism by which growth factor receptors promote the activation of specific intracellular signaling pathways and biological responses and suggest that receptor serine phosphorylation, in addition to receptor tyrosine phosphorylation, may play essential roles in regulating pleiotropic biological responses.

It is noteworthy that the motif encompassing the PLC-γ and 14-3-3 binding sites in FGFR2 demonstrate high sequence homology to the GM-CSF receptor motif encompassing the Shc and 14-3-3 binding sites we described in our earlier studies (17, 18). We therefore performed database searches to identify similar putative phosphotyrosine/phosphoserine "bidentate motifs" in other cell surface receptors. As shown in Table 1, these searches have identified a number of receptors that contain similar conserved phosphotyrosine/phosphoserine bidentate motifs. It is noteworthy that the 14-3-3 binding site at S779 in FGFR1 and FGFR2 is not conserved in FGFR3 and FGFR4. In this regard, it is interesting that FGFR4 is unable to regulate cell proliferation of Ba/F3 cells (66), and FGFR3 is significantly less potent than FGFR1 in promoting PC12 cell differentiation (26). Each motif in Table 1 consists of a conserved putative PTB or SH2 binding site that is followed by a conserved putative 14-3-3 binding site and is found in diverse growth factor, cytokine, and integrin receptors. Thus, our results suggest that such bidentate motifs intersect the activation of distinct molecular pathways downstream of functionally

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diverse signaling proteins to regulate pleiotropic cellular responses.

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