Signal Transduction Pathway of Human Fibroblast Growth Factor Receptor 3

IDENTIFICATION OF A NOVEL 66-kDa PHOSPHOPROTEIN*

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Stimulation of fibroblast growth factor receptor 3 (FGFR3) results in a variety of functional effects, including regulation of epithelial cell growth and differentiation. In order to characterize the signaling pathway through which FGFR3 regulates cell growth, L6 cells lacking any endogenous FGFR were stably transfected with the two different human isoforms, FGFR3 IIIb and FGFR3 IIIc, that result from alternative splicing of exon III of the FGFR3 gene encoding the ligand binding domain. Expression of FGFR3 IIIc in stably transfected L6 cells conferred growth responses to several members of the FGF family including FGF-1, -2, -4, and -6, while FGFR3 IIIb-expressing cells responded only to FGF-1.

Activation of FGFR3 upon ligand binding resulted in activation of mitogen-activated protein kinase pathway. FGFR3 utilizes two different pools of adapter protein GRB2 to link to Ras. Activated FGFR3 predominantly interacts with GRB2-Sos in complex with a previously identified 90-kDa protein and designated protein 80K-H. In addition, 80K-H-GRB2-Sos complex was found to contain a novel 66-kDa protein. Tyrosine phosphorylation of the 66-kDa protein was dependent on ligand activation of FGFR3, suggesting that the 66-kDa protein may play an important role in FGFR3-specific signaling. In addition to this unique pathway, FGFR3 also links to GRB2-Sos complex via the adapter protein Shc. Furthermore, activated FGFR3 was not able to induce dissociation of GRB2-Sos complex following Sos phosphorylation. In summary, FGFR3 signaling pathway utilizes two GRB2-containing complexes; Shc-GRB2-Sos and 80K-Hpp66-GRB2-Sos; these two complexes may alternatively link FGFG3 to mitogen-activated protein kinase. Finally, activated FGFR3 was also found to result in phosphorylation of phospholipase C-γ but reduced phosphorylation of c-Src.

The fibroblast growth factor (FGF) family encompasses at least 10 recognized ligands, which affect a wide variety of biological events including cell growth and differentiation, neurotide outgrowth, embryogenesis, angiogenesis, and wound healing (1–3). The FGFs exert their pleiotropic effects by binding with various specificities to a family of tyrosine kinase-linked cell surface receptors encoded by four separate genes (4). The variety of FGFR family receptors is increased by alternate exon splicing mechanisms, which provide further diversity and ligand specificity through swapping of exons encoding the ligand binding domain (5–9). Among these FGFs, FGFR3, which corresponds to the IIIc variant of FGFRs, was originally isolated from a human K562 cDNA library (10). FGFR3 also has an alternatively spliced form corresponding to the IIIb splice variants of FGFR1 and -2 (7–9).

In previous studies, the FGFR3 IIIb was present in epithelial cell populations, while non-epithelial cell populations express the alternate form FGFR3 IIIc (9). More recently FGFR3 IIIb was found to promote cell proliferation in colonic epithelial cells (11). FGFR3 mRNA has also been reported to be expressed at high levels in cartilage, suggesting a role for FGFR3 in bone development (12). The disruption of the murine FGFR3 gene resulted in severe and progressive bone dysplasia with enhanced and prolonged endochondrial bone growth (13). Thus, FGFR3 appears to mediate negative regulation of bone growth. Recent studies in FGFR3 have focused on mutations in FGFR3, which are associated with several bone abnormalities in humans (14–16). Although some of these mutations result in ligand-independent activation of FGFR3 (17), the signaling pathway of FGFR3 has not yet been clarified.

Binding of cognate ligand to FGFRs has been known to lead to receptor dimerization, followed by the activation of intrinsic kinase activity (18, 19). The latter results in binding to cellular substrates containing Src homology 2 (SH2) domains such as PLC-γ (20). Several other intracellular substrates including Shc, ERK1, ERK2, cortactin, and c-Src have been implicated in FGFR1 and/or FGFR4 signaling pathway (21–27). Recent studies have demonstrated a general pathway of receptor tyrosine kinases in FGFR1 (28). In this pathway, activated FGFR1 interacts with the proximal cytosolic substrate Shc. Tyrrosyl-phosphorylated Shc binds the adapter protein GRB2-nucleotide exchange factor Sos complex linking FGFR1 to the Ras signaling pathway. Several investigators have also demonstrated the involvement of a 90-kDa protein in FGFR1 and keratinocyte growth factor receptor but not in FGFR4 signaling pathway (29–31). This protein, which appears identical to a protein designated 80K-H (32), binds to activated FGFR1 directly and forms a ternary complex with GRB2/Sos. This complex enables FGFR1 to link to Ras signaling pathway (28, 33).

Seven intracellular autophosphorylation sites have been

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1 The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; ERK, extracellular-signal regulated kinase; GST, glutathione S-transferase; GRB2, growth factor receptor-bound protein; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, MAP kinase kinase; PAGE, polyacrylamide gel electrophoresis; PLC-γ, phospholipase C-γ; PVDF, polyvinylidene difluoride.
identified in FGFR1 (20, 33). Some of these are conserved among all known FGFRs (i.e., Tyr^{653}, Tyr^{654}, Tyr^{730}, and Tyr^{766} in FGFR1). Among these, Tyr^{766} in the carboxyl tail of FGFR1 provides a binding site for one of the SH2 domains of PLC-γ (20). Although all known FGFR have a consensus sequence corresponding to Tyr^{766} in FGFR1, PLC-γ phosphorylation caused by activated FGFR4 is considerably weaker than that by FGFR1 (21, 31, 34, 35). Although the stimulation of FGFR1, FGFR3, or FGFR4 results in increased DNA synthesis (8, 36), activated FGFR4 failed to elicit signals characteristic of the FGFR1: tyrosine phosphorylation of Shc and ERK and induction of fos and $tis11$ RNA expression (21).

A recent study demonstrated that FGFR4 was phosphorylated mainly on serine and threonine residues and to a lesser degree on tyrosine residues upon ligand stimulation. Activated FGFR4 but not FGFR1 was associated with 85-kDa serine kinase (35, 37). Thus the subtle differences in cytoplasmic domain of FGFRs may confer different signaling and biological potentials in each FGFRs. Observations that both acidic FGF (aFGF) and basic FGF had a mitogenic effect on rat bladder carcinoma cells and that only aFGF caused cell dissociation and dispersion suggesting that these two biological activities could be mediated through distinct signaling pathways of different receptors (38). In this report we have characterized the intracellular responses elicited by ligand activation of FGFR3 in stably transfected L6 cells.

**MATERIALS AND METHODS**

Reagents—Radiochemicals (γ-32P)ATP, [3H]thymidine, and [3H]thymidine were obtained from DuPont NEN. Human recombinant FGF-1 (aFGF), FGF-2 (basic FGF), FGF-4, FGF-5, FGF-6, and FGF-7 were obtained from R&D Systems. Polyclonal antibodies against FGF, GRB2, SOS, c-Src, Raf-1, ERK1, and ERK2 were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Polyclonal antibodies against PLC-γ and monoclonal for phosphotyrosine (4G10) were from Upstate Biotechnology, Inc., Lake Placid, NY. Polyclonal antibodies against Shc and monochlonal for phosphotyrosine (PY20) were from Transduction Laboratories, Lexington, KY. Polyclonal antibody against the predicted carboxyl terminus of 80K-H was kindly provided by Drs. K. C. Goh and G. R. Guy (Signal Transduction Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, Republic of Singapore). GRB2 full-length glutathione S-transferase (GST) fusion protein conjugated to agarose was obtained from Santa Cruz Biotechnology.

**Expression Vector Construction**—A full-length human cDNA encoding FGFR3 protein was isolated from an oligo(dT)selected HT-29 cDNA plasmid library in CDMS vector, and cloned into pBluescript (Stratagene, La Jolla, CA) (9). To construct FGFR3 IIIb expression vector, a BssHII/KpnI fragment was released from pBluescript containing full-length FGFR3 IIIb cDNA and subsequently ligated into a HindIII/KpnI-digested pcDNA3 plasmid (Invitrogen, San Diego, CA). A cDNA fragment containing the region that encodes the ligand binding domain of FGFR3 IIIc was isolated by reverse transcription-polymerase chain reaction. Poly(A)−-enriched RNA (100 ng) from K-562 cells was reverse transcribed in a 20-μl reaction mixture containing random hexamers according to the manufacturer’s instructions (Perkin Elmer). Polymerase chain reaction was carried out using 10 μl of the reverse transcription cDNA products with the primer pair of 5′-ACCGCATTGGAGCCGATCAAGG2′ and 5′-AGCGTGCATGTCATGCTC-3′ for 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The polymerase chain reaction product was cloned into pCR-Script (Stratagene). A PnuII/MluI fragment was digested and subsequently ligated into a PnuII/MluI-digested pcDNA3 plasmid containing FGFR3 IIIb cDNA. Sequencing was performed by the dideoxy termination method.

**Cell Culture and Transfection**—The L6 rat myoblast cell line devoid of endogenous FGF receptor was grown in modified Eagle medium (Cellgro, Mediatech Inc., Herndon, VA) with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Sigma). Cells were transfected using Lipofectin (Life Technologies, Inc.) with 1 μg of pcDNA3 plasmid containing full-length human cDNA encoding either FGFR3 IIIb or IIIc. Stable transfectants were established by selection in medium supplemented with 800 μg/ml G418 (Life Technologies, Inc.) for 5 weeks. The expression of FGFR3 protein was assessed by Western blotting using anti-FGFR3 antibody, which recognizes the common carboxyl terminus of FGFR3. One cloned cell line from each construct was chosen for further study.

**Metabolic Labeling of Cells and Treatment with Tunicamycin**—Cells were labeled with [35S]methionine at a concentration of 100 μCi/ml. In some experiments, cells were treated with 5 μg/ml tunicamycin (Sigma) for 2 h before and throughout a 3-h [35S]methionine-labeling period. The labeled cells were lysed and subjected to immunoprecipitation using anti-FGFR3 antibody. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

**Preparation of Cell Lysate**—After washing with phosphate-buffered saline (PBS), cells were incubated for 1 h in serum-free medium. After stimulation with FGFs in the presence of heparin (1.25 μg/ml), cells were washed with phosphate-buffered saline and solubilized in lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) containing phosphatase inhibitors (500 μM sodium orthovanadate, 100 μM NaF, and 10 mM sodium pyrophosphate). After 30 min on ice, cell lysates were cleared by centrifugation at 10,000 × g for 20 min. The protein concentration in each sample was quantified by the Bradford method (Bio-Rad).

**Immunoprecipitation and Immunoblot Analysis**—Antibodies to the respective proteins were added to the clarified cell lysates, then incubated for 2 h and precipitated with protein A-Sepharose (Pharmacia Biotech). The resulting immune complexes were washed three times with lysis buffer. SDS-sample buffer was added, and the immune complexes were boiled for 5 min. Samples were electrophoresed through a 7.5% or a 10% polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After saturation in blocking buffer (1 × Tris-buffered saline (TBS), 0.05% Tween 20, 1% bovine serum albumin), the blots were incubated for 4 h room temperature or overnight 4°C with primary antibodies in blocking buffer. After washing in TBS, 0.05% Tween 20, incubation with appropriate secondary antibody diluted in TBS, 0.05% Tween 20 containing 5% nonfat dry milk was performed for 45 min at room temperature, and binding was detected using Renaissance chemiluminescent reagents (DuPont), according to the manufacturer’s instructions. Some immunoblots were stripped of antibodies with 62.5 mM Tris (pH 6.8), 2% SDS-PAGE and autoradiography.

**GST Fusion Protein Binding**—GRB2-GST fusion protein conjugated to agarose was added to clarified cell lysate at a concentration of 5 μg/ml (5-μl bead volume with 5 μg of protein), then incubated for 2 h. After washing in lysis buffer as described above, samples were separated by SDS-PAGE and subjected to immunoblotting.

**Immunoprecipitation Using Complex Kinase**—Anti-Raf1, anti-ERK1, or anti-ERK2 antibody was individually added to the clarified cell lysates, then incubated for 2 h and precipitated with protein A-Sepharose. The immune complexes were washed twice with lysis buffer and once with kinase reaction buffer. The immune complexes were resuspended in 50 μl of kinase buffer supplemented with 10 μg/ml of γ-32P]ATP and 7.5 μg of myelin basic protein (MBP) (Sigma). Kinase reactions were performed for 30 min at 25°C and terminated by boiling in SDS-sample buffer. Samples were analyzed by SDS-PAGE and autoradiography.

**Mitogenic Assay**—10^4 L6 cells expressing FGFR3 were seeded into 96-well plate in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Twelve hours before adding FGFs, cells were washed three times and then cultured for 20 h in serum-free medium containing FGFs in concentrations ranging from 5 to 5000 pg/ml in the presence of heparin (1.25 μg/ml). 1 μCi of [3H]thymidine was added to each well. Cells were harvested after 4 h by filtration through glass fiber filters. Incorporation of radioabeled thymidine was determined using a liquid scintillation counter.

**RESULTS**

**FGFR3 Expression in L6 Cells**—The L6 rat myoblast cell line has been used extensively to study the signaling and biological functions of FGFR because this cell line does not express any endogenous FGFRs. In order to characterize pathways mediating FGFR3 response without the confounding presence of other FGFR, L6 cells were stably transfected with 5 μg/ml pcDNA3 containing full-length cDNA encoding the two isoforms FGFR3 IIb and FGFR3 IIIc. After prolonged culture in selection medium, the transfectants were selected for expression of the FGFR3 by Western blot analysis. Western blotting of protein lysates from cloned cells transfected with FGFR3 IIb using an antibody that recognizes the carboxyl terminus of FGFR3 dem-
onstrated that FGFR3 IIIb protein was indeed present in this clone as a 125-kDa glycoprotein, while IIIc-transfected cells expressed a 135-kDa protein (Fig. 1A). To determine the basis of the molecular weight difference observed between the two forms of FGFR3, FGFR3 IIIb and IIIc were immunoprecipitated after metabolic labeling with [35S]methionine of cells treated with tunicamycin, an inhibitor of N-linked glycosylation. FGFR3 IIIb transfectants yielded a 97-kDa protein, while IIIc yielded a 105-kDa protein (Fig. 1B). In aggregate, Western blotting confirmed successful expression of intact FGFR3s in the stable transfectants.

Ligand-induced Phosphorylation of FGFR3 and Mitogenic Response of L6 Cells Transfected with FGFR3—Ligand specificities of murine FGFR3 have been previously reported (8, 36, 39). Although the region of the third Ig-like domain closest to the transmembrane domain which determines ligand specificity of FGFR3 is highly conserved between mouse and human, there is still some sequence divergence between the FGFs in these two species. After establishing stable transfectants of FGFR3s, subsequent studies were carried out to determine the ligand binding specificity of human FGFR3. L6 cells expressing FGFR3 IIIb or IIIc were stimulated with each of six human recombinant FGFs for 15 min in the presence of 1.25 µg/ml of heparin. After stimulation, cells were lysed and subjected to immunoprecipitation with anti-FGFR antibody. The immune complexes precipitated by anti-FGFR antibody were separated by SDS-PAGE, and autophosphorylation of FGFR3 was analyzed by Western blotting using anti-phosphotyrosine antibodies. As shown in Fig. 2, only FGF-1 induced significant tyrosine phosphorylation of FGFR3 IIIb (Fig. 2A), while FGF-1, FGF-2, FGF-4, and FGF-6 all induced equivalent phosphorylation of FGFR3 IIIc (Fig. 2B).

In parallel studies, the mitogenic response of transfectants to FGFs was assessed. The stable transfectants exhibited a comparable pattern of mitogenic response to FGFs similar to that found in phosphorylation. Thus cells stably expressing FGFR3 IIIb responded only to FGF-1 (Fig. 3A), while cells expressing FGFR3 IIIc responded well to FGF-1, FGF-2, FGF-4, and FGF-6 (Fig. 3B). The non-transfected L6 parent was unresponsive to all FGFs.

Association of Tyrosyl Phosphoproteins with Activated FGFR3—In order to analyze the time course of receptor autophosphorylation, FGFR3-expressing L6 cells were stimulated for 1, 5, and 15 min with 1 nM aFGF in the presence of 1.25 µg/ml heparin. Tyrosyl-phosphorylated proteins in total cell lysate or the immune complex precipitated by anti-FGFR were analyzed by Western blotting using anti-phosphotyrosine antibodies. Stimulation of L6 cells expressing FGFR3 resulted in tyrosine phosphorylation of several proteins including the transduced receptor itself, as well as 175-, 90-, 66-, 44-, and 42-kDa proteins (Fig. 4A). As shown in Fig. 4B, autophosphorylation of FGFR3 can be detected within 1 min of stimulation and is maximal at 15 min. Of note, a tyrosyl-phosphorylated 90-kDa protein was co-precipitated with FGFR3 between 5 and 15 min after stimulation.

Phosphorylation of Shc—To determine the potential role of Shc, a SH2 domain-containing protein that anchors GRB2 to receptor tyrosine kinase (40), phosphorylation of Shc and its binding to FGFR3 were analyzed. After 5 min of stimulation of FGFR3-expressing L6 cells with aFGF, the 52-kDa form of Shc was prominently phosphorylated. The 46-kDa form of Shc was phosphorylated to a lesser degree, but no phosphorylation of the 66-kDa form of Shc was detected. (Fig. 5A). As a control, the same blot was stripped and rehybridized with anti-Shc antibody to verify the equivalent immunoprecipitation of 66-, 52-,
with 1 nM aFGF were separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-phosphotyrosine antibodies. Tyrosyl-phosphorylated proteins were visualized by enhanced chemiluminescence. Molecular weight standards and the position of tyrosyl-phosphorylated proteins are indicated on the left and right, respectively.

Fig. 4. Induction of tyrosine phosphorylation in association with activated FGFR3. Cell lysates (panel A) or lysates immunoprecipitated by anti-FGFR3 antibody (panel B) prepared from L6 cells expressing FGFR3 either unstimulated or stimulated for indicated time with 1 nM aFGF were subjected to Western blotting using anti-phosphotyrosine antibodies. Tyrosyl-phosphorylated proteins were visualized by enhanced chemiluminescence. Molecular weight standards and the position of tyrosyl-phosphorylated proteins are indicated on the left and right, respectively.

Fig. 5. Activated FGFR3 results in a time-dependent phosphorylation of Shc. A, immunoprecipitates by anti-Shc antibody from L6 cells expressing FGFR3 either unstimulated or stimulated for indicated time with 1 nM aFGF were subjected to Western blotting using anti-phosphotyrosine antibodies. B, the same blot was stripped and rehybridized with anti-Shc antibody. Cell lysate was also used as a positive control for the three isoforms of Shc. C, FGFR3 immunoprecipitates from L6 cells expressing FGFR3 either unstimulated or stimulated for 5 min with aFGF were subjected to Western blotting using anti-Shc antibody.

and 46-kDa isoforms of Shc (Fig. 5B).

Subsequently, experiments were carried out to assess the potential binding of Shc to activated FGFR3. Immunoprecipitation using FGFR3 antiserum followed by immunoblotting using Shc antiserum could not demonstrate direct binding of 46- and 52-kDa forms of Shc to activated FGFR3. In contrast, the 66-kDa form of Shc, which was not phosphorylated after stimulation by aFGF, appeared to bind to FGFR3 constitutively (Fig. 5C).

Interaction of GRB2 with Shc and Other Cellular Constituents—To analyze the components interacting with GRB2 in response to FGFR3 activation, lysates from aFGF-stimulated L6 cells expressing FGFR3 were subjected to immunoprecipitation using GRB2 antiserum or GST-GRB2, followed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 6A). GRB2 immunoprecipitates were also subjected to GRB2 immunoblotting, which confirmed that equal amounts of GRB2 were immunoprecipitated (Fig. 6B). The prominent tyrosyl-phosphorylated protein interacting with GRB2 is a 90-kDa protein that appears to be identical to the 90-kDa protein found to bind to activated FGFR3 directly (Fig. 4B). To identify this protein, cell lysate from aFGF-stimulated L6 cells expressing FGFR3 was incubated with GST-GRB2. Material bound to GST-GRB2 was separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies or anti-80K-H antibody, an antibody that recognizes a previously identified 90-kDa phosphoprotein involved in FGFR1 signaling pathway (32). Immunoblot with anti-phosphotyrosine antibodies resulted in localization of the same tyrosine phosphorylation as found after immunoblotting of anti-GRB2 immunoprecipitates (data not shown). Immunoblotting with anti-80K-H antibody confirmed the identity of the 90-kDa protein (Fig. 6C).

In addition to the 90-kDa (80K-H) protein, 66- and 52-kDa tyrosyl-phosphorylated proteins were found to interact with GRB2. It is likely that the 52-kDa component of Shc on the basis of its molecular weight and binding to GRB2. To demonstrate in vivo binding of Shc to GRB2, GRB2 immunoprecipitates were subjected to Shc immunoblotting. As shown in Fig. 6D, the binding of 52-kDa component of Shc to GRB2 was detectable within 5 min after aFGF stimulation. The interaction of Shc with GRB2 was also demonstrated by immunoblotting of anti-Shc antibody immunoprecipitates from aFGF-stimulated L6 cells with anti-GRB2 antibody (Fig. 6E). As expected, there was no detectable GRB2 protein in anti-Shc antibody immunoprecipitates without aFGF stimulation, since Shc was not phosphorylated. However, the binding of GRB2 to Shc can be seen within 5 min after stimulation (Fig. 6E). In contrast, the 66-kDa protein does not appear to correspond to previously identified phosphoproteins involved in signal transduction. As demonstrated in Fig. 6D, this phosphoprotein does not represent the 66-kDa form of Shc. In separate experiments, this component was not recognized by antisera against c-Src, p62, and protein-tyrosine phosphatase 1D/Syp (data not shown).

Interaction of GRB2/Sos Complex with Shc and Other Cellular Constituents—Further studies were carried out to analyze guanylnucleotide exchange factor Sos phosphorylation and its interaction with Shc, GRB2 and other proteins. In L6 cells expressing FGFR3, treatment with aFGF resulted in a characteristic alteration in Sos mobility (Fig. 7B). Previous studies reported Sos phosphorylation after insulin stimulation resulted in its dissociation from GRB2 (41–43). In contrast, EGF stim-
Fig. 7. Participation of Sos in FGFR3 signal transduction. A, cell lysates from L6 cells expressing FGFR3 either unstimulated or stimulated for indicated time with aFGF were subjected to immunoprecipitation using anti-Sos antibody. Immunoprecipitates were subjected to Western blotting using anti-phosphotyrosine antibodies (B) or anti-GRB2 antibody (C). The same cell lysate as prepared in panels B and C were subjected to immunoprecipitation using anti-GRB2 antibody (D) or anti-Shc antibody (E) followed by Western blotting using anti-Sos antibody.

Fig. 8. Activated FGFR3 leads to phosphorylation of Raf-1 and mitogen-activated protein kinase. A, cell lysate from L6 cells expressing FGFR3 either unstimulated or stimulated for indicated time with aFGF were subjected to immunoprecipitation using indicated antibodies. Immunoprecipitates were subjected to kinase assay in the presence of 10 μCi of [γ-32P]ATP and 7.5 μg of MBP. Kinase reaction was performed for 30 min at 25°C and samples were analyzed by SDS-PAGE and autoradiography. B, immunoprecipitates obtained in panel A were subjected to Western blotting using anti-phosphotyrosine antibodies (upper panel). The same membranes were stripped and immunoblotted with anti-ERK1 or ERK2 antibody, respectively (lower panel).

ulation does not induce Sos dissociation from GRB2 but instead Shc dissociation from GRB2:Sos complex (44, 45). GRB2 immunoblotting of the same blot used in Fig. 7B demonstrated that equivalent GRB2 amounts co-immunoprecipitated with Sos after aFGF stimulation, indicating the persistence of GRB-Sos complex (Fig. 7C). Furthermore, Sos immunoprecipitation of GRB2 immunoprecipitates clearly demonstrated that Sos remains complexed with GRB2 despite phosphorylation reflected by a characteristic reduction in electrophoretic mobility (Fig. 7D). In addition, anti-phosphotyrosine immunoblotting of Sos immunoprecipitates demonstrated the coupling of the 90-kDa (80K-H) and 66-kDa phosphorylated proteins to Sos following aFGF stimulation (Fig. 7E). Of note, the time course of these two phosphorylated protein is identical to that observed in anti-phosphotyrosine immunoblotting of GRB2 immunoprecipitates (Fig. 6A). Collectively, these data indicated that GRB2 and Sos remain complexed after FGFR3 activation.

Following demonstration of the interaction of Shc with GRB2 in FGFR3-expressing cells upon receptor activation by aFGF (Fig. 6, D and E), Shc immunoprecipitates were subjected to Sos immunoblotting to determine the potential interaction of Shc with the GRB2:Sos complex. As shown in Fig. 7E, aFGF stimulation resulted in co-immunoprecipitation of Sos with Shc, indicating the presence of the ternary complex of Shc-GRB2-Sos. The amounts of Sos co-immunoprecipitated with Shc after aFGF stimulation remained unchanged over the observed time period.

Activated FGFR3 Lead to Raf-1 and Mitogen-activated Protein Kinase—Next, the phosphorylation of the serine/threonine kinase Raf-1 and mitogen-activated protein kinase was assessed. Activation of Raf-1 was assessed by in vitro phosphorylation of MBP (Fig. 8A), and the phosphorylation of ERK1 and ERK2 was evaluated by in vitro phosphorylation of MBP (Fig. 8A), as well as immunoblotting with anti-phosphotyrosine antibody for ERK1 or ERK2 immunoprecipitates (Fig. 8B). Phosphorylation of ERK1 and ERK2 was induced within 5 min after aFGF stimulation and persisted for at least 15 min. Indeed, the prominent tyrosyl-phosphorylated proteins of 42 and 44 kDa detected in total cell lysate (Fig. 4A) were identified as ERK1 and ERK2, respectively, on the basis of their molecular weight and recognition by anti-ERK antibodies.

Activated FGFR3 Results in PLC-γ Phosphorylation but Reduced Phosphorylation of c-Src—Tyr766 in the carboxyl-terminal tail of FGFR1 has been shown to provide a binding site for the SH2 domain of PLC-γ (20). Since FGFR3 also has the consensus sequence Tyr766-Leu-Asp-Leu corresponding to Tyr766-Leu-Asp-Leu in FGFR1, phosphorylation of PLC-γ in response to aFGF stimulation was analyzed. Anti-phosphotyrosine immunoblotting of PLC-γ immunoprecipitates from aFGF-stimulated FGFR3-expressing L6 cells showed that maximum phosphorylation of PLC-γ was detected at 1 min after stimulation with aFGF and persisted for 15 min (Fig. 9A). The equal amount of PLC-γ in each immunoprecipitate was confirmed by rehybridizing the same membrane used in Fig. 9A with anti-PLC-γ antibody (Fig. 9B). We also tried to demonstrate the binding of PLC-γ to activated FGFR3. However, immunoblotting of anti-PLC-γ antibody immunoprecipitates from aFGF-stimulated L6 cells expressing FGFR3 did not reveal any FGFR3 in these complex (data not shown).

Members of the Src family of cytoplasmic tyrosine kinase are known to associate with FGFR (24–27). c-Src in FGFR3-expressing cells is constitutively phosphorylated, and the level of phosphorylation was reduced within 1 min after aFGF stimulation (Fig. 9C). c-Src immunoblotting of c-Src immunoprecipitates confirmed equal amounts of c-Src at each time point (Fig.
In this study, we have characterized the ligand specificities of human FGFR3s and the downstream signaling pathway elicited following activation by the cognate ligand aFGF. To achieve these goals, cells expressing each of the two isoforms of FGFR3 were established by stable transfection with plasmid pcDNA3 containing full-length cDNA encoding either of the two isoforms of FGFR3, designated FGFR3 IIIb and FGFR3 IIIc. Although the size of the two proteins should be essentially identical on the basis of amino acid sequence, the expressed receptor isoforms differ in molecular weight. This difference (IIIb = 125 kDa, IIIc = 135 kDa) appears to be due to N- and/or O-linked glycosylation, since in vitro translated protein of FGFR3 IIIb and IIIc exhibited the same molecular weight in Western blotting using the same antibody (data not shown), and the difference in two isoforms expressed by transfected L6 cells partially persists after treatment with the N-linked glycosylation inhibitor tunicamycin. Comparison of amino acid sequences of the third Ig-like domain of the two isoforms of FGFR3 reveals an increased number of potential N- and/or O-linked glycosylation site in IIIc (9), consistent with this speculation.

The biologically relevant ligands for FGFR3 have not been defined. Recently, paired interactions between the nine known ligands and the major splice forms of four known murine FGFRs have been reported (36). Murine FGFR3 IIIb was found to be activated by FGF-1 (aFGF) and FGF-9; while FGFR3 IIIc was activated by FGF-1, -2, -4, -8, and -9 (8, 36, 46). We have examined the ability of six available human recombinant FGFs (FGF-1, -2, -4, -5, -6, and -7) to activate the two isoforms of human FGFR3, as assessed by both ligand-induced autophosphorylation of the receptor and the mitogenic response of cells stably transfected with FGFR3 IIIb or IIIc. In contrast to previously reported results with murine FGFR3, our data demonstrate that FGF-6 is able to induce the phosphorylation of FGFR3 IIIc and is able to stimulate L6 cells expressing FGFR3 IIIc to proliferate. The mitogenic activity of FGF-6 in FGFR3 IIIc-expressing L6 cells is almost equal to that of FGF-2 or -4. Although the amino acid identity between mouse and human FGF-6 is more than 90%, the subtle difference in sequence between these two species might confer different ligand specificity of the receptor.

Signaling pathways utilized by the FGFR family have mainly been studied in FGFR1 and FGFR4, but that of FGFR3 has not been well characterized. In the present studies, we demonstrate the linking of activated FGFR3 to a similar MAP kinase pathway. Activation of receptor tyrosine kinase upon ligand binding results in providing binding sites by cellular substrates containing SH2 domains such as PLC-γ, Shc, and GRB2 (47). The 52-kDa and 46-kDa components of Shc were phosphorylated in response to FGFR3 activation. However, we were unable to demonstrate any direct interaction of activated FGFR3 with phosphorylated Shc, despite conservation of the potential Shc binding site on FGFR3 (Tyr556 and Tyr728) corresponding to Tyr550 and Tyr760 on FGFR1, respectively (4, 46). Additionally, phosphorylation of Shc was reported to be dependent on Tyr550 in FGFR1 corresponding to Tyr756 in FGFR3 (28). In contrast, tyrosine phosphorylation of Shc in response to EGF is known to be largely independent of individual auto-phosphorylation sites and occurs even when several or all auto-phosphorylation sites have been deleted from the receptor (49, 50). These observations suggest that the interaction between FGFR3 and Shc may be weak or indirect.

The adapter protein GRB2 functions to couple signals from receptor tyrosine kinase to Ras by recruiting the nucleotide exchange factor, Sos, to the plasma membrane (51–53). As expected, we could not demonstrate the direct binding of the adapter protein GRB2 to activated FGFR3 (data not shown), with the absence of any consensus binding site for GRB2 in FGFR3. Instead, we have noted binding of tyrosine-phosphorylated Shc, 90- and 66-kDa proteins to GRB2/Sos complex following FGFR3 activation. Since the 90- and 66-kDa proteins are detectable in GRB2 or Sos immunoprecipitates, but not in Shc immunoprecipitates, it is plausible that these two proteins utilize a different pool of GRB2 than the GRB2 that binds to Shc (Fig. 10). The 90-kDa protein appears to be identical to a recently identified GRB2 binding protein defined by anti-
80K-H antibody. Although we were unable to detect direct binding of 80K-H to activated FGFR3, it is likely that the prominent tyrosyl-phosphorylated protein co-precipitated with activated FGFR3 upon ligand binding is identical to the 90-kDa protein recognized by 80K-H antisera (32). This observation suggests that protein 80K-H functions to couple signals from FGFR3 to Ras via a GRB2-Sos complex. Tyrosine phosphorylation of the 66-kDa (pp66) protein was seen immediately after 1 min of aFGF stimulation, which suggests that this protein is phosphorylated directly by activated FGFR3. This component was not recognized by antisera against c-Src, protein-tyrosine phosphatase 1D/Syp, or pp62.

Thus, the tyrosine phosphorylation of receptor and/or Shc results in the formation of a ternary complex (i.e., Shc-GRB2-Sos) that targets Sos to the plasma membrane location of Ras (54, 55). In this manner, Sos can effect the exchange of GDP for GTP on Ras. Once in the activated GTP-bound state, Ras associates with members of the Raf family of serine/threonine kinases (56, 57). Inactivation of Ras following insulin stimulation appears to result from feedback serine/threonine phosphorylation of Sos by the Ras/Raf/MEK/ERK pathway. This phosphorylation event correlates directly with dissociation of the GRB2-Sos complex and the return of GTP-bound Ras to the GDP-bound state (41, 42, 43, 58). EGF also induces serine/threonine phosphorylation of Sos and transient activation of Ras. However, EGF stimulation does not result in a decrease amount of GRB2 bound to Sos (44, 45). EGF receptor directly associates with GRB2-Sos complex upon ligand stimulation. In contrast the insulin receptor does not directly associate with GRB2-Sos but interacts indirectly through docking proteins such as Shc.

FGFR3 downstream signaling resembles that of insulin utilizing Shc as a docking protein in the signaling pathway from the receptor to Ras. Although ligand-induced FGFR3 activation resulted in Sos phosphorylation and ERK activation, activation of FGFR3 did not induce dissociation of GRB2-Sos complex following Sos phosphorylation. One unique feature of FGFR3 signal pathway is the utilization of two GRB2-containing complexes (i.e., Shc-GRB2-Sos and 80K-H-pp66-GRB2-Sos), which can link FGFR3 to Ras. It is unlikely that Shc-GRB2-Sos remain in a ternary complex following Sos phosphorylation. The amount of GRB2 in Shc immunoprecipitates is substantially less than that in Sos immunoprecipitates. GRB2 immunoprecipitated with Shc may represent the amount of GRB2 in the ternary complex of Shc-GRB2-Sos (Fig. 6E). On the other hand, GRB2 immunoprecipitated with Sos may reflect the amount of GRB2 in both ternary complexes of Shc-GRB2-Sos and 80K-H-pp66-GRB2-Sos (Fig. 7C). In aggregate, these findings suggest that 80K-H-pp66-GRB2-Sos may be predominant in linking FGFR3 to Ras. In this dominant complex, Sos does not dissociate from GRB2, so that it overcomes the effect of the minor Shc-GRB2-Sos complex.

The serine/threonine kinase Raf-1 functions downstream of Ras in a signal transduction cascade, which transmits mitogenic stimuli from the plasma membrane to the nucleus. Raf-1 phosphorylates the dual specific kinase MEK, which in turn phosphorylates ERK1 and 2 (53, 59). FGFR3 activation resulted in Raf-1 phosphorylation, and phosphorylation and activation of ERK1 and ERK2 in a manner parallel to that observed on other receptor tyrosine kinases. Although the level of Raf-1 phosphorylation in FGFR3-expressing cells after ligand stimulation is low, only low levels of active Raf-1 are needed to activate the signaling cascade (60).

Consistent with previous studies in FGFR1 or FGFR4 and conservation of a binding site for PLC-γ in the carboxyl tail of FGFR3 (Tyr760), aFGF stimulation of L6 cells expressing human FGFR3 results in PLC-γ phosphorylation. Although we were not able to detect direct binding of PLC-γ to activated FGFR3, it is likely that the phosphorylation of PLC-γ following FGFR3 activation might be mediated by a consensus binding motif of Tyr760-Leu-Asp-Leu in FGFR3.

c-Src, a member of a family of cytosolic tyrosine kinase, has been reported to be involved in signal transduction mediated by FGFs (24–27). Stimulation of NIH 3T3 cells with aFGF resulted in increased kinase activity of Src (26). In contrast, in porcine aortic endothelial cells and lung fibroblasts from Chinese hamster, activation of FGFR1 caused reduced autophosphorylation of Src (27). Thus, the effects of Src kinase activity transduced by FGFs appear to be cell- and/or receptor-specific. Our observation of down-regulated Src autophosphorylation following aFGF stimulation of FGFR3-expressing L6 cells suggesting that activated FGFR3 may exert a negative effect on Src kinase activity.

The present studies demonstrate that activated FGFR3 utilizes a MAP kinase pathway similar to that reported on FGFR1 (28). FGFR3 shares about 70% amino acid sequence identity with FGFR1 in its cytoplasmic domain, but three of the seven intracellular autophosphorylation sites identified in FGFR1 are not conserved in FGFR3 (i.e., Tyr463, Tyr583, and Tyr585) (33). Although the differences in cytoplasmic domain of FGFR3 from FGFR1 are subtle, it appears that FGFR3 has a signaling pathway that could confer biological responses distinct from other FGFRs. More importantly, activated FGFR3 results in tyrosine phosphorylation of a novel 66-kDa protein, which binds to GRB2. This protein interacts with activated FGFR3 directly, suggesting that the 66-kDa component may play an important role in a FGFR3-specific signaling pathway.

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