Interaction of Fibroblast Growth Factor Receptor 3 and the Adapter Protein SH2-B:

A Role in Stat5 Activation

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SUMMARY

Fibroblast growth factor receptor 3 (FGFR3) influences a diverse array of biological processes, including cell growth, differentiation and migration. Activating mutations in FGFR3 are associated with multiple myeloma, cervical carcinoma and bladder cancer. To identify proteins that interact with FGFR3 and which may mediate FGFR3-dependent signaling, a yeast two-hybrid screen was employed using the cytoplasmic kinase domain of FGFR3 as the bait. We identified the adapter protein SH2-B as an FGFR3-interacting protein. Coimmunoprecipitation experiments demonstrate binding of the SH2-Bβ isoform to FGFR3 in 293T cells. Tyrosine phosphorylation of SH2-Bβ was observed when coexpressed with activated FGFR3 mutants, such as the weakly activated mutant N540K or the strongly activated mutant K650E, both associated with human developmental syndromes. The extent of tyrosine phosphorylation of SH2-Bβ correlates with receptor activation, suggesting that FGFR3 activation mediates tyrosine phosphorylation of SH2-Bβ. Furthermore, two tyrosine phosphorylation sites of FGFR3, Y724 and Y760, are required for optimal binding of the Src homology-2 (SH2) domain of SH2-Bβ. We also demonstrate the phosphorylation and nuclear translocation of Stat5 by activated FGFR3, which increases in response to overexpression of SH2-Bβ. Taken together, our results identify SH2-Bβ as a novel FGFR3 binding partner which mediates signal transduction.
INTRODUCTION

Fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases (RTKs) that integrate many different intercellular signals affecting cell growth, differentiation, migration, wound healing and angiogenesis, depending on the target cell type and developmental stage (1,2). The FGFR family comprises of four structurally related members: FGFR1, FGFR2, FGFR3 and FGFR4, exhibiting three extracellular immunoglobulin-like (Ig) domains, a single transmembrane domain and a split intracellular tyrosine kinase domain (3,4,5). Mutations in FGFRs, which may be either familial or spontaneous in origin, are responsible for a large number of human developmental disorders including skeletal dwarfism and craniosynostosis syndromes (6,7,8). Translocations and mutations affecting members of the FGFR family are also importantly associated with several human cancers (6,9-12).

FGFR3 plays a particularly important role in skeletal development (13-15). Disruption of murine FGFR3 produces severe and progressive bone dysplasia with enhanced endochondral bone growth, suggesting that FGFR3 mediates the negative regulation of bone growth (16,17). Mutations in FGFR3 are directly responsible for human dwarfism syndromes, including hypochondroplasia, achondroplasia and thanatophoric dysplasia (TD) (6). Several of the mutations that cause these syndromes reside within the FGFR3 kinase domain, and result in varying degrees of constitutive receptor activation. The N540K substitution, located proximal to the split tyrosine kinase domain, underlies the mild skeletal dwarfism hypochondroplasia, and confers weak constitutive activation (15,18). At the other end of the spectrum, the K650E substitution located within the activation loop of the kinase domain, relieves the normal requirement for regulatory phosphorylation at Y647 and Y648 and leads to profound constitutive
kinase activation in comparison to wild-type FGFR3 (19). This mutation causes thanatophoric
dysplasia type II (TDII), a neonatal lethal dwarfism syndrome (6,19). Recently, a different
activating substitution at this same position, K650M, has been associated with the syndrome
SADDAN, or Severe Achondroplasia with Delayed Development and Acanthosis Nigricans
(12).

Abnormal activation of FGFR3 as a result of somatic mutation has been reported in
conjunction with several human cancers, including multiple myeloma, cervical carcinoma, and
bladder carcinoma (7,8,20). The specific FGFR3 mutations involved include K650E and K650M
in the kinase domain, or R248C, S249C, G370C and Y373C, in the extracellular domain. All of
these mutations identified in human neoplasia have been previously described as activating
mutations associated with TDI, TDII or SADDAN (6,19,21,22).

In the presence of heparin sulfate proteoglycan, fibroblast growth factors (FGFs) bind to
FGFRs, causing receptor dimerization and autophosphorylation of tyrosine residues (23-25).
These phosphotyrosine residues provide specific binding sites for signaling proteins containing
Src homology 2 (SH2) domains or phosphotyrosine-binding (PTB) domains (26,27). For
example, Y766 in FGFR1 has been shown to interact with phospholipase C-γ (PLC-γ) (28). The
adapter protein fibroblast growth factor receptor substrate 2 (FRS2) has also been shown to
associate with FGFR1 (29-31). Activation of FGFR1 leads to tyrosine phosphorylation of FRS2
at several sites, leading to recruitment of Grb2 (32). Besides PLC-γ and FRS2, little is known
about substrates of FGFRs that lead to mitogenesis and differentiation.

Given the importance of understanding FGFR3-mediated signaling, both for human
developmental syndromes and also for those human cancers where FGFR3 activation has been
observed, we wished to identify novel FGFR3-interacting proteins that may represent important substrates for downstream signaling. Towards this end, we employed a yeast two-hybrid screen in which the bait was the kinase domain from either wild-type FGFR3, or from an activated mutant. Using the weakly activated N540K mutant as the bait, we were able to identify four candidate binding proteins that interact with FGFR3, one of which is the adapter protein SH2-B.

SH2-B contains several protein-protein interaction motifs, including a pleckstrin homology (PH) domain, a SH2 domain and multiple proline-rich regions (33,34). At least three splice variants of SH2-B (α, β, and γ) have been identified that have identical N-terminal and SH2 domains, but differ in their C-terminal domains (33,35,36). SH2-B has previously been shown to interact with other RTKs including platelet-derived growth factor (PDGF) receptor, insulin receptor, and TrkA receptor as well as the non-receptor tyrosine kinase Janus kinase 2 (JAK2) (33-35,37-44). Since SH2-Bβ has been the most studied isoform of SH2-B, we used SH2-Bβ to further characterize the interaction with FGFR3 described here. In PC12 cells, tyrosine-phosphorylated SH2-Bβ binds to Grb2 and is sufficient to mediate nerve growth factor (NGF) induction of Ras and mitogen-activated protein kinase (MAPK) (38,45,46). SH2-Bβ has also been demonstrated to bind to JAK2, to stimulate the kinase activity of JAK2, and to increase tyrosine phosphorylation of Stat3 and Stat5B when coexpressed with JAK2 (35,37).

In this study, we have identified and characterized SH2-B as an FGFR3 binding partner. We also demonstrate that activated FGFR3 can directly phosphorylate SH2-Bβ. In addition, we show that expression of SH2-Bβ together with activated FGFR3 increases Stat5B phosphorylation in 293T cells. Stat5B was also observed to relocalize exclusively to the nucleus.
upon FGFR3-mediated signaling through SH2-Bβ. Our data thus suggest that the adapter protein SH2-Bβ may represent an important signaling molecule that mediates downstream biological effects of FGFR3 activation.
**EXPERIMENTAL PROCEDURES**

**Plasmid Construction** - Full-length myc-epitope tagged SH2-Bβ and GFP-Stat5B were generous gifts from C. Carter-Su (35,47). FGFR3-wild-type (R3-WT), FGFR3-K650E (R3-K650E), and myristylated FGFR3-K650E (myr-R3-K650E) clones were described previously (19,22,48). The FGFR3-N540K (R3-N540K) mutant was constructed from R3-WT by using QuikChange site-directed mutagenesis (Stratagene). LexA-R3-WT, LexA-R3-N540K and LexA-R3-K650E were constructed through the insertion of the cytoplasmic domain of R3-WT, R3-N540K and R3-K650E, respectively, into the LexA-fusion vector pBTM116, which was constructed by P. Bartel and S. Fields. To construct the myc-epitope tagged SH2 domain of SH2-B (myc-SH2B), pVP16-SH2-B isolated from the yeast two-hybrid screen was digested at flanking BamHI and EcoRI sites, and the resulting restriction fragment was subcloned into BglII/EcoRI digested pCS3+MT vector. GST-SH2 was generated by subcloning myc-SH2 into the vector pGEX-KG (Pharmacia). The Y to F mutants were constructed as described previously (49), and subcloned into pBTM116. The myc-SH2-Bβ(R555E) was made as described previously (37).

**Yeast Two-Hybrid Screen** - A yeast two-hybrid screen was performed according to previously published protocols (50,51). The two-hybrid plasmids pBTM116, pVP16 and LexA-lamin were kindly provided by S. Hollenberg and J.A. Cooper (Fred Hutchinson Cancer Research Center). LexA-R3-WT, LexA-R3-N540K, or LexA-R3-K650E constructs were cotransformed with a 9.5 dpc mouse embryonic cDNA library fused to pVP16 into the L40 strain of *Saccharomyces cerevisiae*. Transformants were selected on His† medium for 3-4 days at

8
30°C. The resulting colonies were subjected to the filter-lift color assay and tested for β-galactosidase activity (50). Potential positive clones were selected, and prey plasmids containing library cDNA inserts were isolated and shuttled into *E. coli* HB101 cells. Positives were further confirmed by testing pVP16-cDNA against LexA-lamin, and were sequenced by the Center for AIDS Research Molecular Biology Core, University of California San Diego. The NCBI BLAST program was used to determine the identity of positive clones.

**Immunoprecipitation and Immunoblot** - 293T cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in 10% CO₂. Sub-confluent cells were transfected with 10 µg of DNA by calcium phosphate precipitation (52). Two days after transfection, cells were harvested and lysed in 0.5% NP-40 lysis buffer {20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 µg/mL aprotinin, and 10 µg/mL leupeptin}. Lysates were pre-cleared with 40 µl of 50% Protein A-Sepharose beads, and then incubated with 2 µg of polyclonal FGFR3 (C-15) (Santa Cruz Biotechnology) antibody or monoclonal myc (9E10) antibody (Santa Cruz Biotechnology) overnight at 4°C. Protein A-Sepharose beads were then added for at least two hours and the immunoprecipitated samples were washed 3 times with lysis buffer, boiled 3 min in sample buffer, and analyzed by 10% SDS-PAGE. For experiments not requiring immunoprecipitation, lysates were analyzed by 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were immunoblotted with FGFR3 (C-15) antibody, myc (9E10) antibody, phosphotyrosine (4G10) antibody (Upstate Biotechnology), followed by enhanced chemiluminescence (ECL) (Amersham). To reprobe with other antibodies, membranes were
stripped of bound antibodies in stripping buffer {100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8)} and incubated for 30 min at 60°C with rotation.

For endogenous SH2-Bβ association with FGFR3, NIH3T3 cells were transfected with 15 µg pcDNA3 or R3-K650E. Two days post-transfection, cells were harvested and lysed with 0.1% NP-40 lysis buffer, and 1.5 mg of pre-cleared protein were immunoprecipitated with SH2-Bβ antibody generously provided by Dr. Carter-Su (35) at 4°C overnight. Protein A-Sepharose beads were then added for at least two hours and the immunoprecipitated samples were washed 3 times with lysis buffer, boiled 3 min in sample buffer, and analyzed by 10% SDS-PAGE. Proteins were then transferred to Immobilon-P membrane and probed with FGFR3 (C-15) antibody. The membrane was then stripped and probed with SH2-Bβ antibody followed by ECL.

For Stat5 phosphorylation experiments, 5 µg of the indicated constructs were transfected into 293T cells. Cells were lysed in 1% NP-40 lysis buffer, and 40 µg of lysate was analyzed by 10% SDS-PAGE and transferred to Immobilon-P membrane. The membrane was then probed with phospho-Stat5 antibody (Cell Signaling) and proteins were detected by ECL. The membrane was then stripped and reprobed with Stat5 (C-17, Santa Cruz Biotechnology), FGFR3 (C-15), and myc (9E10) antisera to ensure equal levels of protein expression.

*Glutathione S-transferase (GST) Fusion Proteins* - Bacteria transformed with GST-SH2 or GST plasmid were grown overnight and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) the next day. Bacterial pellets were resuspended in 20 ml of NETN {100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% vol/vol Nonidet P-40 with 2 mM PMSF, 15
µg/mL aprotinin, 20 µg/mL leupeptin, and 20 µg/mL pepstatin A}. Cell suspensions were then sonicated, followed by centrifugation at 20 krpm for 30 min. Supernatants were added to GST beads and rotated at 4°C overnight. Beads then were washed five times with NETN and boiled in sample buffer.

_Baculovirus expression of R3-K650E -_ The kinase domain isolated from full-length R3-K650E (aa 457-827) was subcloned into pFASTBAC Htc (Life Technologies), which contains a histidine tag and elements necessary for cloning and subsequent transfer to the baculovirus genome. Purification was as previously described (53).

_In Vitro Kinase Assay -_ 15 µl of GST-fusion proteins were washed once in kinase buffer {20 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 5 mM MgCl₂} and were subsequently incubated with or without 15 µl of baculovirus-expressed R3-K650E in 30 µl kinase buffer plus 20 µCi [³²P]-γ-ATP at 37°C for 20 min. Samples were then washed with NETN five times, boiled in sample buffer, resolved by 7.5% SDS-PAGE, transferred to membrane, and visualized by autoradiography. The membrane was then immunoblotted with GST (Z-5) antibody (Santa Cruz Biotechnology) and detected by ECL.

_Liquid Culture β-Galactosidase Assay -_ Yeast were cotransformed with the indicated plasmids and grown on His↑ plates for 3-4 days at 30°C. Liquid culture β-galactosidase assays were then performed according to the protocol provided by Clontech using o-Nitrophenyl β-D-galactopyranoside (ONPG) (Sigma). Each reaction was carried out at 30°C until the sample
became yellow. Samples that did not develop a yellow color were stopped at the end of the fourth hour. The absorbance of each sample was measured at 420 nm, and β-galactosidase activity was calculated using the following formula: β-galactosidase units = 1000 x OD_{420}/(t x V x OD_{600}), where t is elapsed time (min) of incubation, V is 0.1 ml x concentration factor, and OD_{600} is the absorbance of 1 ml culture at 600 nm. The data obtained were the results of five independent experiments.

*Immunofluorescence* - 2C4 cells were cultured in 10% heat-inactivated FBS containing 80 µg/ml G418 in 5% CO₂. Cells were plated into 60 mm plates containing glass coverslips at a density of 5 x 10⁵. The next day, the cells were transfected with a total of 2.1 µg DNA of the indicated constructs using Effectene (Qiagen) according to the manufacturer’s directions. DNA ratios for the triple transfection were: 0.1 µg GFP-Stat5B, 0.5 µg FGFR3, and 1.5 µg myc-SH2-Bβ. pcDNA3 was added to make up the difference in the single and double transfections. Twenty-four hours post-transfection, the coverslips were fixed with 3% paraformaldehyde. To visualize protein localization, the cells were permeabilized with 0.5% Triton-X, rinsed with PBS, and blocked with 3% BSA for 30 min. The cells were incubated with FGFR3 antibody (1:500) for 1 hour, washed, then incubated with 1:500 rhodamine-conjugated anti-rabbit secondary antibody for 45 min. After washing with PBS, the coverslips were mounted onto glass slides with 90% glycerol in 0.1 M Tris-HCl (pH 8.5) plus phenylenediamine to prevent fading. Cells were photographed using a Nikon Microphot-FXA microscope with a Hamamatsu C5810 camera.
RESULTS

Identification of an FGFR3-Interacting Protein - A yeast two-hybrid screen was employed to identify potential substrates of FGFR3. To construct the bait for the two-hybrid screen, the entire intracellular domain of FGFR3 was fused to the LexA DNA-binding domain. Three different bait constructs were utilized which differed in the extent of constitutive FGFR3 kinase activation (Figure 1A): i) LexA-R3-WT; ii) LexA-R3-N540K, incorporating the weakly activated N540K mutation that causes hypochondroplasia (15,18); and iii) LexA-R3-K650E, incorporating the strongly activated K650E mutation that causes TDII (19).

These constructs were used as two-hybrid baits and screened against a 9.5 dpc mouse embryonic cDNA library. Cotransformed yeast were plated on His↑ plates, and the activation of the HIS3 reporter gene was used to select for protein-protein interactions. The LexA-R3-WT bait resulted in ~50 positives, but none yielded identifiable clones. The LexA-R3-K650E bait appeared toxic to the yeast in the large scale transformation, and no positives resulted from the screen. Only one of the bait proteins, LexA-R3-N540K, yielded significant positive clones in the screen. Approximately 150 potential positive clones were isolated and, of these, 50 clones scored positive on the filter-lift β-galactosidase assay. False positives were further eliminated by testing against LexA-lamin, and 25 clones remained positive. One of the positive clones identified was SH2-B.

The cDNA insert of the SH2-B plasmid identified is 342 bp in length and corresponds to residues 518-631. The region includes the entire SH2 domain plus a small portion of flanking sequences as shown in Figure 1B. We also wished to determine if the LexA-R3-WT and LexA-R3-K650E constructs would interact with the SH2-B clone, and performed a small scale yeast
transformation. Both LexA-R3-WT and LexA-R3-K650E were found to interact with the SH2
domain of SH2-B (Table 1). PLC-γ, which was also isolated from the screen, was used as a
positive control since it has been shown to bind to Y766 of FGFR1, which corresponds to Y760
in FGFR3 (28). This yeast two-hybrid interaction demonstrates for the first time a direct
interaction between FGFR3 and PLC-γ. The empty pVP16 vector was used as an additional
negative control. Thus, we demonstrate a novel interaction between the SH2 domain of SH2-B
and FGFR3.

The SH2 Domain of SH2-Bβ Mediates Interaction with FGFR3 in vivo - To confirm
that the SH2 domain alone can interact with FGFR3 in mammalian cells, a myc-epitope tagged
derivative of SH2-Bβ was constructed containing only the SH2 domain. This derivative,
designated myc-SH2, was transfected into 293T cells in the absence or presence of full-length
FGFR3-wild-type (R3-WT), FGFR3-N540K (R3-N540K) or FGFR3-K650E (R3-K650E). Cell lysates were immunoprecipitated with FGFR3 antibody, resolved by 10% SDS-PAGE, and
immunoblotted with myc antisera. The results indicate that the SH2 domain alone is sufficient to
bind to activated FGFR3 (Figure 2, lanes 3 and 4). Significant association was observed between
myc-SH2 and the strongly activated mutant, R3-K650E (Figure 2, lane 4), while little or no R3-
WT was recovered in association with myc-SH2 (Figure 2, lane 2). In comparison, intermediate
association was seen between R3-N540K and myc-SH2. These results suggest that the binding
of the SH2 domain to FGFR3 may be dependent on the extent of receptor activation.
Immunoblotting with FGFR3 antibody shows the expression of the FGFR3 derivatives
immunoprecipitated from transfected cell lysates (Figure 2, middle panel), and equivalent
expression of myc-SH2 was confirmed by analyzing lysate alone (Figure 2, bottom panel).
Endogenous SH2-Bβ Interacts with Activated FGFR3 - We next wished to determine whether endogenous mouse SH2-Bβ would interact with transfected FGFR3 in NIH3T3 cells. Cells were transfected with either mock or R3-K650E, lysed, and immunoprecipitated with SH2-Bβ antisera. Lysates and immunoprecipitated samples were then analyzed by 10% SDS-PAGE, followed by immunoblotting with FGFR3 or SH2-Bβ antisera. The top panel of Figure 3 demonstrates the presence of activated FGFR3 in the SH2-Bβ-immunoprecipitated sample (lane 4). Activated FGFR3 does not bind to Protein A-Sepharose beads alone, as demonstrated in lane 6 of the top panel. The bottom panel of Figure 3 confirms the presence of endogenous mouse SH2-Bβ in the lysate and SH2-Bβ-immunoprecipitated lanes (lanes 1-4), but not in the Protein A-Sepharose plus lysate lanes (lanes 5 and 6).

Full-Length SH2-Bβ Interacts with FGFR3 - Myc-epitope tagged full-length SH2-Bβ (myc-SH2-Bβ) (35) was examined for its ability to bind to FGFR3 derivatives in mammalian cells. 293T cells were transfected with empty pcDNA3 vector, or with myc-SH2-Bβ in the absence or presence of full-length R3-WT, R3-N540K or R3-K650E. Cell lysates were immunoprecipitated with myc antibody, resolved by 10% SDS-PAGE, and immunoblotted with FGFR3 antisera. All three of the FGFR3 derivatives bound to myc-SH2-Bβ, as demonstrated by the recovery of FGFR3 proteins in the myc-SH2-Bβ immunoprecipitates (Figure 4). The extent of FGFR3 recovery correlated generally with the magnitude of receptor activation. The strongly activated R3-K650E mutant exhibited the strongest association (Figure 4, lane 6, top panel), the weakly activated R3-N540K mutant was recovered to a lesser extent (Figure 4, lane 5, top panel), while R3-WT showed the lowest association (Figure 4, lane 4, top panel). The membrane
was stripped and reprobed with myc antibody to confirm the expression of myc-SH2-Bβ (Figure 4, middle panel). Equivalent expression of FGFR3 was also confirmed by analyzing lysate alone (Figure 4, bottom panel). These results demonstrate that SH2-Bβ forms a complex with FGFR3 in vivo that can be recovered by immunoprecipitation, and that the extent of SH2-Bβ/FGFR3 complex formation correlates with the level of FGFR3 activation.

**FGFR3 Activation Promotes Tyrosine Phosphorylation of SH2-Bβ** - SH2-Bβ has been shown previously to be tyrosine phosphorylated in response to growth hormone, nerve growth factor, and PDGF (33,34,37-40). To determine whether FGFR3 activation promotes tyrosine phosphorylation of SH2-Bβ, 293T cells were transfected with empty pcDNA3 vector, or with myc-SH2-Bβ in the absence or presence of R3-WT, R3-N540K or R3-K650E. Lysates were immunoprecipitated with myc antibody and resolved by 10% SDS-PAGE. Immunoblotting with 4G10 phosphotyrosine antisera revealed that the tyrosine phosphorylated form of SH2-Bβ specifically associated with activated FGFR3 (Figure 5A, lanes 5 and 6 of the top panel). Tyrosine phosphorylated SH2-Bβ associated strongly with R3-K650E (Figure 5A, lane 6, top panel), but much less so with R3-N540K (Figure 5A, lane 5, top panel). R3-WT was unable to stimulate the tyrosine phosphorylation on SH2-Bβ (Figure 5A, lane 4, top panel). The presence of SH2-Bβ was confirmed by reprobing the stripped membrane with myc antibody (Figure 5A, middle panel). Equivalent FGFR3 expression was confirmed by immunoblotting the lysates with FGFR3 antibody (Figure 5A, bottom panel).

Since both FGFR3 and SH2-Bβ migrate between 116 kDa and 97.5 kDa on 10% SDS-PAGE, we wanted to rule out the possibility that the bands shown in the top panel of Figure 5A
were tyrosine phosphorylated FGFR3. To accomplish this, we utilized a truncated R3-K650E construct which contained only the intracellular domain and a myristylation signal at the N-terminus (myr-R3-K650E) to properly localize the protein to the plasma membrane, and which we have extensively characterized in previous studies (22,48). This construct runs at ~50 kDa. 293T cells were transfected with empty pcDNA3 vector, or with myc-SH2-Bβ in the absence or presence of myr-R3-K650E. The samples were then lysed and immunoprecipitated with myc antibody. Immunoblotting with 4G10 phosphotyrosine antibody showed that SH2-Bβ is indeed tyrosine phosphorylated (Figure 5B, lane 4, top panel), which confirmed that activated FGFR3 stimulates tyrosine phosphorylation of SH2-Bβ. The bottom panel of Figure 5B shows that myc-SH2-Bβ was expressed in the appropriate samples (lanes 2 and 4).

Activated FGFR3 phosphorylates the SH2 domain of SH2-B - We then examined whether the kinase domain of activated FGFR3 directly phosphorylated SH2-B by performing in vitro kinase assays. A GST fusion protein containing the SH2 region isolated from the yeast two-hybrid screen (GST-SH2) was constructed. We prepared baculovirus-expressed R3-K650E and added it to immobilized GST or GST-SH2 in the presence of γ[^32P]ATP. Samples were resolved by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and followed by autoradiography. A high level of GST-SH2 phosphorylation was detected only when the activated kinase domain was present (Figure 6, lane 4 of the left panel), demonstrating that the SH2 domain of SH2-B can be directly phosphorylated by the kinase domain of FGFR3. To confirm that the phosphorylated protein was GST-SH2, the membrane was probed with GST antibody (Figure 6, right panel). These data suggest that activated FGFR3 can phosphorylate the
SH2 domain of SH2-B, although we cannot completely exclude the possibility that another kinase may have copurified with the baculovirus-expressed R3-K650E protein.

Identification of SH2-B Binding Sites in FGFR3 - Seven autophosphorylation sites in FGFR1 have been described previously (23,28). Based on the sequence alignment with FGFR1, the potential autophosphorylation sites (Y577, Y647, Y648, Y724, and Y760) in FGFR3 were identified. In addition, Y770 in FGFR3 is conserved throughout FGFRs, suggesting that it may be a potential autophosphorylation site as well. A schematic diagram of the intracellular domain of FGFR3 with six potential autophosphorylation sites is shown in Figure 7A. Y647 and Y648 are located in the activation loop, and their phosphorylation is involved in the conformational changes that accompany receptor activation (49). Previously we showed that substitution of all non-activation loop Y residues with F caused FGFR3 to be inactive (49). We therefore focused on residues Y724, Y760, and Y770. To determine the tyrosine residue(s) of FGFR3 required for SH2-B binding, we generated a series of LexA-R3-N540K mutants containing phosphorylation-site mutations in which the tyrosine residues were mutated to phenylalanine. LexA-R3-N540K was used as the template for these mutants (Figure 7B), since this derivative was used as bait in the initial yeast two-hybrid screen.

Each mutant was tested against the SH2-B region isolated from the screen by β-galactosidase liquid assay (Table 2). PLC-γ was used as a positive control due to its previously characterized interaction with FGFR1 (28). Among the mutants, LexA-770F exhibited the strongest interaction with SH2-B (Table 2). This mutant lacks Y770, but retains Y724 and Y760. This suggests that one or the other, or both, of these residues is important for the
interaction with SH2-B. The LexA-724F mutant, which retains Y760 and Y770 but lacks Y724, showed a decrease in binding. In addition, the LexA-760F mutant, which retains Y724 and Y770 but lacks Y760, also exhibited a significantly decreased interaction. These data indicate that Y724 and Y760 are important for SH2-B binding.

The results presented in Table 2 also demonstrate that LexA-R3-N540K exhibits significant interaction with PLC-γ. Interestingly, when Y770 was removed by mutation, the resulting mutant LexA-770F exhibited increased interaction with PLC-γ, and to a lesser extent with SH2-B. This observation could suggest that Y770 plays a negative regulatory role in FGFR3 signaling, at least with regard to signaling through the effector proteins SH2-B and PLC-γ.

Activated FGFR3 and SH2-Bβ Increases Stat5 Phosphorylation and Activation - A previous study has shown that GH-induced binding of SH2-B and JAK2 results in enhanced tyrosine phosphorylation of cellular proteins, including Stats (37). Thus, we next examined whether SH2-Bβ and activated FGFR3 affected Stat signaling. 293T cells were transfected with empty pcDNA3 vector, R3-WT, R3-K650E, R3-K650E plus myc-SH2-Bβ, or R3-K650E plus SH2-Bβ(R555E). The SH2-Bβ(R555E) mutant contains a mutation within the SH2 domain of SH2-Bβ to prevent binding by SH2-domain binding substrates (37). Lysates were then analyzed by 10% SDS-PAGE, transferred to a membrane, and immunoblotted with phospho-Stat5 antisera. As demonstrated in Figure 8A, activated FGFR3 alone induces a small amount of endogenous Stat5 phosphorylation (lane 3). When SH2-Bβ is coexpressed with activated FGFR3, there is a significant increase in Stat5 phosphorylation (Figure 8A, lane 4). In contrast,
coexpression of the SH2-Bβ mutant containing a defective SH2 domain with activated FGFR3 resulted in a decrease in endogenous Stat5 phosphorylation (Figure 8A, lane 5). The membrane was then stripped and reprobed with Stat5 (Figure 8B) to demonstrate equivalent levels of protein in each sample. The membrane was further stripped and reprobed with myc antibody to show expression of transfected myc-SH2-Bβ (Figure 8C), and then with FGFR3 antibody to show expression of transfected FGFR3 (Figure 8D).

Following tyrosine phosphorylation at the C-terminus, Stat proteins undergo dimerization and relocalization from the cytoplasm to the nucleus (54,55). We examined whether Stat5 localization was dependent upon expression of SH2-Bβ and activated FGFR3. We utilized a human fibroblast cell line, 2C4, for transfection with the indicated constructs. To ensure that each transfected cell contained all the indicated constructs, 0.1 µg GFP-Stat5B was cotransfected with 0.5 µg FGFR3-K650E or 0.5 µg each of FGFR3-K650E and myc-SH2-Bβ (wild-type or R555E mutant). Each GFP-Stat5B positive cell examined should then contain FGFR3-K650E and, in the case of the triple transfection, both FGFR3-K650E and myc-SH2-Bβ (wild-type or R555E mutant). Thus, in some cases, cells not exhibiting GFP-Stat5B staining could contain FGFR3-K650E as shown in Figure 9, panel j.

When GFP-Stat5B (47) was cotransfected with wild-type FGFR3, Stat5B was predominantly cytoplasmic (Figure 9, panel e). Cotransfection of GFP-Stat5B with the activating mutant, R3-K650E, resulted in an increase in nuclear localization of Stat5B (Figure 9, panel h). When SH2-Bβ was also transfected with R3-K650E and GFP-Stat5B, Stat5B relocalized completely into the nucleus (Figure 9, panel k). In the presence of the mutant SH2-Bβ(R555E), R3-K650E and GFP-Stat5B, however, Stat5B was localized to the cytoplasm (Figure
9, panel n). These data correlate with the increase in phosphorylation observed in Figure 8 upon coexpression of activated FGFR3 and SH2-Bβ, and a decrease in phosphorylation upon coexpression of activated FGFR3 and SH2-Bβ (R555E).
DISCUSSION

Numerous skeletal and developmental disorders have been shown to result from mutations in FGFRs (6,56,57). Mutations in FGFR3 result in many human disorders, including TDI, TDII, SADDAN, and dwarfism (6,21). More recently, FGFR3 has been linked to cancers such as multiple myeloma and bladder and cervical carcinoma (7,8). Unlike other RTKs, few of the immediate downstream signals of FGFR3 have been identified. In this study, we have identified SH2-B as an FGFR3 binding protein using the yeast two-hybrid screen. Coimmunoprecipitation experiments in 293T cells between FGFR3 and SH2-B confirmed this interaction. We have also found by β-galactosidase liquid assay that Y724 and Y760 in FGFR3 interact with the SH2 domain of SH2-B. In addition, activated FGFR3 promotes binding to SH2-Bβ and stimulates tyrosine phosphorylation of SH2-Bβ. Furthermore, the kinase domain of FGFR3-K650E can directly phosphorylate SH2-B in vitro. These results suggest that SH2-B is a direct substrate of FGFR3.

Previously, PLC-γ represented the only SH2 domain-containing binding partner for FGFRs (28). The SH2-B clone isolated from the yeast two-hybrid screen primarily contains the SH2 domain (Figure 1B), demonstrating that SH2-B binds to FGFR3 via its SH2 domain. Consistent with this, the SH2 domain alone was sufficient to bind to activated FGFR3 in mammalian cells. Since SH2 domains have been found to bind to phosphotyrosine residues (58), it is likely that SH2-B interacts with the phosphotyrosine residue(s) of FGFR3. Coimmunoprecipitation experiments demonstrated that binding of the SH2-Bβ isoform to FGFR3 correlated with receptor activation, which further supports that the interaction is phosphotyrosine dependent. This also corresponds to the fact that a N540K mutation leads to a
milder form of activated FGFR3 than a K650E mutation. Interestingly, our data indicate that full-length SH2-Bβ can also weakly associate with wild-type FGFR3 (Figure 4). One possible explanation for this observation is the existence of an additional low-affinity binding site(s) in SH2-B for FGFR3. In fact, SH2-Bβ has been shown to bind to tyrosine phosphorylated JAK2 not only via its SH2 domain but also by its N-terminal region (amino acids 1 to 555); however, this binding is not phosphotyrosine-dependent (59).

We also show that both Y724 and Y760 of FGFR3 are required for interaction with the SH2 domain of SH2-B. Mutating these residues to phenylalanine impairs the association between FGFR3 and the SH2 domain. Previous studies from our laboratory have shown that a derivative of FGFR3 containing all conserved tyrosine residues stimulated transformation, Stat activation, and phosphatidylinositol (PI) 3-kinase activation (49). Substitution of all non-activation loop tyrosine residues with phenylalanine rendered the FGFR3 derivative inactive; however, the addition of Y724 restored its ability to stimulate the above signaling pathways (49). Data from our two-hybrid screen also indicate that the SH2 domain of the p85 regulatory subunit of PI 3-kinase interacts with FGFR3 (data not shown). It is possible that the SH2 domains of p85 and SH-2B compete for binding of Y724 in FGFR3, resulting in the activation of different signaling pathways. We have also previously shown that both Y724 and Y760 were required for maximal Stat activation (49). Similarly, both tyrosine residues may be necessary to mediate the signaling events carried out by SH2-B in response to FGFR3 activation. Nonetheless, the possibility that the SH2 domain interacts with only Y724 or Y760 should not be excluded.

We also demonstrate by an in vitro kinase assay that the SH2 domain of SH2-Bβ can be
directly phosphorylated by activated FGFR3. Since SH2-Bβ has 9 tyrosine residues, some of these residues may be directly phosphorylated by FGFR3 while others may be phosphorylated by non-receptor tyrosine kinases such as JAK2 (35). Among these residues, Y439, Y494, and Y624 were predicted to be potential tyrosine phosphorylation sites (33). The Y624 residue in particular is located within a YVPS motif, which is a putative phosphorylation site by PDGF receptor (33). Since the SH2-B clone isolated from the screen contains the entire SH2 domain as well as the Y624 residue, it would be interesting to determine if FGFR3 specifically phosphorylates Y624. There are two other tyrosine residues, Y525 and Y564, in the SH2 domain of SH2-Bβ which may also serve as phosphorylation sites. Future studies will determine which residue(s) is specifically phosphorylated by FGFR3. Identification of the tyrosine residues phosphorylated by FGFR3 will be important in studying the signaling pathway(s) mediated by SH2-Bβ as they will suggest molecular mechanisms of recruitment of signaling proteins by FGFR3 activation via specific tyrosine phosphorylation.

In this study, we have characterized a novel interaction between FGFR3 and SH2-Bβ and we have demonstrated that FGFR3 activation results in the tyrosine phosphorylation of SH2-Bβ. We also demonstrate one mechanism by which FGFR3 mediates downstream signaling. In 293T cells, we observe phosphorylation of Stat5 in cells transfected with activated FGFR3. This phosphorylation increases in response to expression of SH2-Bβ. We also demonstrate the nuclear translocalization of Stat5B when both activated FGFR3 and SH2-Bβ are coexpressed. When the mutant SH2-Bβ(R555E) was coexpressed with activated FGFR3, Stat5B becomes inactivated and is predominantly cytoplasmic. SH2-B therefore appears to represent a novel and biologically relevant substrate of FGFR3.
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FOOTNOTES

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2 The abbreviations used are: FGFR, fibroblast growth factor receptor; SH2, src homology 2; Grb2, growth factor receptor-bound 2; PDGF, platelet-derived growth factor; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinase; Stat, signal transducer and activator of transcription; PLC-γ, phospholipase C-gamma; RTKs, receptor tyrosine kinases; TD, thanatophoric dysplasia; FRS2, fibroblast growth factor receptor substrate 2; PH, pleckstrin homology; TrkA, Tropomyosin receptor kinase A; PI 3, phosphatidylinositol 3; SADDAN, Severe Achondroplasia with Delayed Development and Acanthosis Nigricans

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FIGURE LEGENDS

Figure 1. **FGFR3 interacts with SH2-B in a two-hybrid screen.** (A) Schematic representation of the LexA-FGFR3 baits. TM (shaded) represents the transmembrane domain and Ig represents an immunoglobulin-like domain. The black region represents the LexA DNA-binding domain. (B) The SH2 domain of SH2-B was isolated from the two-hybrid screen. Full-length rat SH2-Bβ is 670 amino acid long containing 9 tyrosine residues. The black regions represent proline-rich regions, PH represents the pleckstrin homology domain, and SH2 represents the src homology 2 domain. The expanded region shown was isolated from the yeast two-hybrid screen is mouse SH-2B (Gen Bank Ac. No. AF036355).

Figure 2. **The SH2 domain of SH2-B interacts with FGFR3 in vivo.** 293T cells were transfected with the indicated constructs, where mock is pcDNA3 vector alone. Cell lysates were immunoprecipitated (IP) with FGFR3 antibody, analyzed by 10% SDS-PAGE, and immunoblotted (IB) with myc antibody (top panel). The middle panel shows the presence of FGFR3 using FGFR3 antibody. Equal expression of the myc-SH2 domain of SH2-B was confirmed by resolving lysate on a 10% SDS-PAGE followed by immunoblotting with myc antibody (bottom panel).

Figure 3. **FGFR3 interacts with endogenous SH2-B.** NIH3T3 cells were transfected with the indicated constructs, lysed, and immunoprecipitated (IP) with SH2-Bβ antibody (lanes 3 and 4) or Protein A-Sepharose beads alone (lanes 5 and 6). Samples were then analyzed by 10% SDS-
PAGE, and immunoblotted (IB) with FGFR3 antibody (top panel). The membrane was then stripped and reprobed with SH2-Bβ antibody to confirm the presence of SH2-Bβ (bottom panel).

**Figure 4. Full-length SH2-Bβ interacts with FGFR3 in vivo.** 293T cells were transfected with the indicated constructs. Cell lysates were immunoprecipitated (IP) with myc antibody, analyzed by 10% SDS-PAGE, and immunoblotted (IB) with FGFR3 antibody (top panel). The membrane was stripped and reprobed with myc antibody to confirm the presence of myc-SH2-Bβ (middle panel). Equal expression of FGFR3 was confirmed by resolving lysate on a 10% SDS-PAGE followed by immunoblotting with FGFR3 antibody.

**Figure 5. Activated FGFR3 tyrosine phosphorylates SH2-Bβ.** (A) 293T cells were transfected with the indicated constructs. Cell lysates were immunoprecipitated (IP) with myc antibody, analyzed on a 10% SDS-PAGE, and immunoblotted (IB) with phosphotyrosine (4G10) antibody (top panel). The membrane was stripped and reprobed with myc antibody to confirm the presence of myc-SH2-Bβ (middle panel). Equal expression of FGFR3 was confirmed by resolving lysate on 10% SDS-PAGE followed by immunoblotting (IB) with FGFR3 antibody (bottom panel). (B) 293T cells were transfected with the indicated constructs, where myr represents the myristylated derivative of R3. Lysates were immunoprecipitated with myc antibody followed by immunoblotting with 4G10 antibody (top panel). The membrane was stripped and reprobed with myc antibody to confirm the expression of myc-SH2-Bβ.
Figure 6. Activated FGFR3 phosphorylates the SH2 domain of SH2-B in vitro. GST or GST-SH2 was incubated with or without the baculovirus-expressed kinase domain of R3-K650E in the presence of 20 μCi of γ-[32P]ATP. Samples were resolved on a 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The left panel shows a 20 min autoradiography. The membrane was then immunoblotted with GST antibody to confirm the expression of GST and GST-SH2 (right panel).

Figure 7. Representation of FGFR3 mutants. (A) A schematic diagram of the intracellular domain of FGFR3 with six potential tyrosine phosphorylation sites (Y577, Y647, Y648, Y724, Y760, and Y770). Y647 and Y648 are crucial for kinase activity. (B) Table of FGFR3 tyrosine mutants used to determine SH2-B binding sites. Mutations were made starting with the LexA-R3-N540K construct, where Y724, Y760, and/or Y770 were mutated from Y to F.

Figure 8. Stat5 activation by FGFR3 and SH2-Bβ. (A) 293T cells were transfected with the indicated constructs. Lysates were analyzed by 10% SDS-PAGE and immunoblotted for phospho-Stat5. (B) The membrane was stripped and reprobed with Stat5 antiserum. (C) Immunoblotting with myc-antibody of the stripped membrane indicated the presence of transfected myc-SH2-Bβ. (D) The membrane was stripped once more and probed with FGFR3 antibody to confirm the presence of FGFR3.

Figure 9. Stat5 relocalizes to the nucleus upon FGFR3-mediated signaling. 2C4 cells were transfected with the indicated constructs. Panel e demonstrates the cytoplasmic localization of
GFP-Stat5B in the presence of R3-WT. When activated R3-K650E is cotransfected with GFP-Stat5B, Stat5B is both cytoplasmic and nuclear as shown in panel h. Upon cotransfection of SH2-Bβ with R3-K650E and GFP-Stat5B, GFP-Stat5B is exclusively nuclear (panel k). When SH2-Bβ(R555E) is coexpressed with R3-K650E and GFP-Stat5B, GFP-Stat5B becomes cytoplasmic. Panels a, d, g, j, and m confirm expression of FGFR3. Panels c, f, i, l, and o represent the nuclei visualized by Hoechst 33342.
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Figure 1

FGFR3

Ig-1  Ig-2  Ig-3

TM  Kinase  Kinase

LexA-R3-WT

LexA  Kinase  Kinase

LexA-R3-N540K

Kinase  N540K  Kinase

LexA-R3-K650E

Kinase  Kinase  K650E

B.

SH2-Bβ

47  55

Y  Y

354

Y

439

Y

494

Y

525

Y

564

Y

624

Y

649

mouse SH2-B (aa 518-631) region isolated from the two-hybrid screen

GDQPLSGYPWFHGLRLSKAAQLV

LEGGTGHGVFLVRSEQSETRGEYV

LTFNFQGKAKHRSHSLNEEGQCRV

QHLWFQSIFDMLEHFRVHPLESQ

SSDVVLSVSYVPSQRQQ
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Figure 2

|       | Mock | R3-WT | R3-N540K | R3-K650E |
|-------|------|-------|----------|----------|
| myc-SH2: | -    | +     | +        | +        |
|       | 1    | 2     | 3        | 4        |

IP: α-FGFR3
IB: α-myc

lysates
IB: α-myc

myc-SH2

39

FGFR3

IP: α-FGFR3
IB: α-FGFR3

myc-SH2
Figure 3

IB: α-FGFR3

IB: α-SH2-Bβ
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Figure 4
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Figure 5A

A

| myc-SH2-Bβ: | - | + | - | + | + | + |
|------------|---|---|---|---|---|---|
|            | 1 | 2 | 3 | 4 | 5 | 6 |

116 -
97.5 -

\{ P-myc-SH2-Bβ \}

IP: α-myc
IB: α-phosphotyrosine

\[ \text{myc-SH2-Bβ} \]

IP: α-myc
IB: α-myc

lysates
IB: α-FGFR3

\[ \text{FGFR3} \]
Figure 5B

- myc-SH2-Bβ:
  - Mock: -
  - myr-R3: -
  - myr-R3-K650E: +
  - 1 2 3 4

- P-myc-SH2-Bβ
- 116 -
- 97.5 -

- IP: α-myc
- IB: α-phosphotyrosine

- myc-SH2-Bβ
- IP: α-myc
- IB: α-myc
| R3-K650E: | - | + | - | + | - | + | - | + |
|-----------|---|---|---|---|---|---|---|---|
| 1 2 3 4   |   |   |   |   |   |   |   |   |

**Kinase Assay**

**IB: α-GST**

- GST
- GST-SH2

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Figure 6
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Figure 7

A

B

| Protein                     | 540 | 724 | 760 | 770 |
|-----------------------------|-----|-----|-----|-----|
| LexA-R3-N540K               | K   | Y   | Y   | Y   |
| LexA-724F                   | K   | F   | Y   | Y   |
| LexA-760F/770F              | K   | Y   | F   | F   |
| LexA-760F                   | K   | Y   | F   | Y   |
| LexA-724F/770F              | K   | F   | Y   | F   |
| LexA-770F                   | K   | Y   | Y   | F   |
| LexA-724F/760F              | K   | F   | F   | Y   |
| LexA-724F/760F/770F         | K   | F   | F   | F   |
Figure 8

A

IB: α-p-Stat5

B

IB: α-Stat5

C

IB: α-myc

D

IB: α-FGFR3
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Figure 9

|            | FGFR3 | GFP-Stat5B | Hoechst |
|------------|-------|------------|---------|
| **Mock**   | ![a](image) | ![b](image) | ![c](image) |
| **R3-WT**  | ![d](image) | ![e](image) | ![f](image) |
| + GFP-Stat5B | ![g](image) | ![h](image) | ![i](image) |
| **R3-K650E** | ![j](image) | ![k](image) | ![l](image) |
| + GFP-Stat5B | ![m](image) | ![n](image) | ![o](image) |
| + SH2-Bβ   | ![j](image) | ![k](image) | ![l](image) |
| **R3-K650E** | ![m](image) | ![n](image) | ![o](image) |
| + GFP-Stat5B | ![m](image) | ![n](image) | ![o](image) |
| + SH2-Bβ(R555E) | ![m](image) | ![n](image) | ![o](image) |
Table 1: Summary of Yeast Two-Hybrid Interactions

| Baits           | PLC-γ | SH2-B | Vp16 vector |
|-----------------|-------|-------|-------------|
| LexA-R3-N540K   | +     | +     | -           |
| LexA-R3-WT      | +     | +     | -           |
| LexA-R3-K650E   | +     | +     | -           |
| LexA-lamin      | -     | -     | -           |

The LexA-R3-N540K bait isolated SH2-B from the two-hybrid screen. SH2-B was then tested against LexA-R3-Wt and LexA-R3-K650E. As a positive control, PLC-γ was tested against each bait. As negative controls, the baits were tested against the Vp16 vector alone and the isolated clones were tested against LexA-lamin. Transformed yeast were grown at 30 °C for 3-4 days on His<sup>+</sup> plates.
### Table 2. β-Galactosidase Assay for SH2-B Binding Sites in FGFR3

| Baits                  | Interaction with |          |          |
|------------------------|------------------|----------|----------|
|                        | PLC$_{\gamma}$   | SH2-B    |          |
| LexA-724F              | 5 ± 1             | 0.4 ± 0.1|          |
| LexA-760F/770F         | 0.1 ± 0.1         | 0.2 ± 0.1|          |
| LexA-760F              | 0.1 ± 0.1         | 0.2 ± 0.1|          |
| LexA-724F/770F         | 4 ± 0.7           | 0.3 ± 0.1|          |
| LexA-770F              | 40 ± 5            | 3 ± 0.1  |          |
| LexA-724F/760F         | 0.2 ± 0.0         | 0.2 ± 0.1|          |
| LexA-724F/760F/770F    | 0.1 ± 0.1         | 0.2 ± 0.1|          |
| LexA-FGFR3(N540K)      | 30 ± 3            | 2 ± 0.4  |          |
| LexA-Lamin             | <0.1              | <0.1     |          |

Mutants were constructed by mutation of tyrosine to phenylalanine in the intracellular domain of FGFR3-N540K, followed by subcloning into the LexA yeast vector pBTM116. Yeast were cotransformed with the indicated plasmids and grown under HIS$^+$ medium at 30°C for 3-4 days prior to β-galactosidase assay. One unit of β-galactosidase is defined as the amount which hydrolyzes 1 mmol of o-nitrophenyl β-D-galactopyranoside (ONPG) per min per cell. Results shown are the mean +/- standard deviation of 5 independent experiments.
Interaction of fibroblast growth factor receptor 3 and the adapter protein SH2-B: A role in Stat5 activation
Monica Kong, Ching S. Wang and Daniel J. Donoghue

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